

# HANDBOOK OF MICROSCOPICAL TECHNIQUE

*For Workers in Animal and Plant Tissues*

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## PREFACE TO THE SECOND EDITION

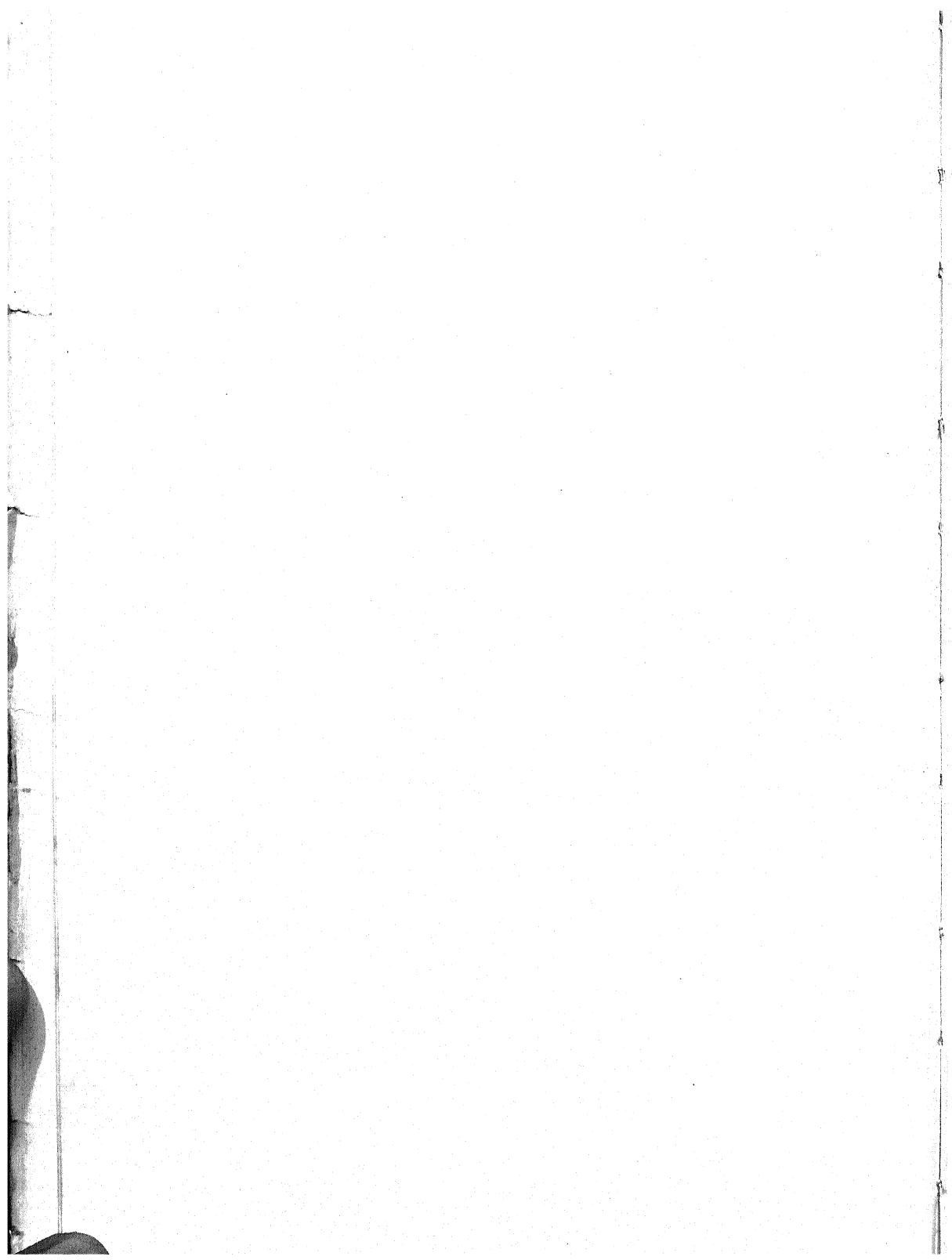
The exhaustion of the first edition of this book seems to indicate the desirability of issuing it in an improved form. Accordingly a strong effort has been made both to bring methods up to date and to add descriptions of new methods and apparatus. Among the additions are a complete, new dioxan technique for paraffin sections, directions for free hand manipulations of living material, methods for staining boutons terminaux, an account of the fused quartz rod method of illuminating living structure, a description of the microincineration method, a presentation of the centrifuge microscope and a description of fluorescent microscopy. The increase in the number of pages from 495 to 698 is an indication of the extent of new material added. There are also a number of new illustrations provided better to present the structure of pieces of apparatus and manipulation procedures. Particularly the index has been much improved.

Once more I have to express appreciation for the understanding and helpful cooperation of the contributors to this volume, and for the invaluable assistance of Dr. H. Irene Corey and of my daughter Mrs. Ruth M. Jones. My wife again lent her welcome assistance in the drudgery of proofreading.

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Philadelphia, Pa.

*March, 1937.*



## PREFACE TO THE FIRST EDITION

In two previous texts, *General Cytology*<sup>1</sup> and *Special Cytology*,<sup>2</sup> edited by Dr. Cowdry, experts in various fields of biology have presented the results of their investigations. In the present work, which extends the series, the *methods* involved in these and similar studies are given.

There are two general needs to be met by a work of this sort. The first is that of the inexperienced worker who requires specific directions which he may apply with confidence to the general run of material, with the expectation of getting desired results. (Part I of this book outlines such standard methods.)

The other requirement is that of the experienced investigator who seeks the latest approved methods for the accomplishment of special technical results. (Part II presents this type of material.) In order to meet such a need as this, involving, as it does, the intimate knowledge of varied and often involved processes, the method of cooperation employed in the above-mentioned books is utilized. Workers highly experienced in the use of specialized methods describe these in detail. Since such directions will be consulted under terms which apply to the particular method or material it seems best to arrange these in appropriate chapters so that they may be reached directly as a group. In a discussion of these special methods, by a system of cross references, given in the text, to the first part of the work, unnecessary repetitions of the descriptions applying to general processes are often avoided. Also through the cross references between these special articles it is possible to follow out completely the methods which can be applied to any particular type of material.

The individual chapters and sections have been left largely as they were sent in by their authors, and this has resulted in some variations of statement extending sometimes even to open contradictions. So long as our procedures have only an empirical basis, judgments will continue to vary.

If one might draw a moral after reviewing, in an editorial capacity, the stated details of microscopical technology, it would be to the effect that while responsible for tremendous advances in our knowledge of living things, it is sadly in need of exact scientific bases for its own dicta. For precision and definiteness of results, microtechnique is now, and probably always will be, largely dependent upon individual skill, patience and

<sup>1</sup> *General Cytology*. Ed. by E. V. Cowdry, Univ. Chicago Press, 1924.

<sup>2</sup> *Special Cytology*. Ed. by E. V. Cowdry, N. Y., Hoeber, Ed. 2, 1933, 3 Vols.

judgment, but until we know just why a fixative or a stain produces a given result under one set of conditions and a different one under others, our interpretations lack an essential element of authority. There is much to be hoped from the present interest of chemists and physicists in biological matters, if they will submit their conclusions to the review of those intimately familiar with the conditions under which organisms live and operate.

Because this is a handbook for practical use there is no historical treatment and generally only such a bibliography as is necessary to complete an understanding of indicated methods. It is assumed that the book will be of interest particularly to workers in bacteriology, botany, cytology, embryology, histology and pathology, but the presentation of general methods in Part I makes it useful to any student of microscopic anatomy.

Limitations of space make it impossible to describe the instruments and apparatus required for microscopical studies, but those interested will find in the work by Simon H. Gage<sup>3</sup> full information on the subject. For the same reason there are few indications given regarding the source of biological materials, but in Guyer's "Animal Micrology"<sup>4</sup> there are numerous good directions of this character. In the encyclopaedic work edited by R. Krause<sup>5</sup> there are exhaustive discussions of many topics briefly treated in the present work. The Vade-Mecum of Lee<sup>6</sup> is a standard reference. For a detailed review of the chemical basis underlying micro-technical processes the work by Gustav Mann<sup>7</sup> is very helpful.

It is a pleasure to acknowledge here the cordial cooperation of all the contributors, which has much lightened editorial drudgery. To Dr. E. V. Cowdry I am much indebted for consulting with the publishers, during my absence from the country, while the book was in press. For much expert bibliographic and secretarial assistance I have to thank my assistant, Miss H. Irene Corey. For reading the proof of the entire book I am under obligations to my wife, to my daughter, Mrs. Ruth M. Thompson, and to Miss Corey. Finally it is a pleasure to acknowledge the cordial and sympathetic cooperation of Mr. Hoeber and his assistants.

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PHILADELPHIA, PA.

*October, 1928.*

<sup>3</sup> Gage, S. H. *The Microscope*. Dark field ed. Ithaca, N. Y., 1925.

<sup>4</sup> Guyer, M. F. *Animal Micrology*. Ed. 2, Chicago, 1917.

<sup>5</sup> Krause, R. *Enzyklopädie der mikroskopischen Technik*. Ed. 3, Berlin, 1927.

<sup>6</sup> Lee, A. B. *The Microtomists' Vade-Mecum*. Ed. 9, London, 1928.

<sup>7</sup> Mann, G. *Physiological Histology*. Oxford, 1902.

# INTRODUCTION

Judged by the quality of the preparations used as a basis for many investigations, the importance of technical processes is not fully appreciated. The value of the results obtained from any microscopical study is directly dependent upon the quality of the technique employed in preparing the material. For this reason it is of utmost importance that every precaution be taken to secure the most accurate preservation attainable of normal conditions. It is unfortunately true, also, that no matter how highly developed a technique may be, faults in its application may entirely invalidate the results. It is not possible, therefore, by any mere statement of a process, to insure a high quality in the results of its use since so much is dependent upon the judgment and experience of the person employing the method.

However, it is feasible, by a clear statement of the steps involved, and of the results obtained by others, to indicate with some definiteness the course of procedure which should be followed. If the beginner could submit the results of his operations to one experienced with the method he would get definite suggestions of the highest value. In most cases the older worker, if convinced that the novice is really in earnest in his efforts, would give him sufficient of his time and experience to set him on the right way.

In any method the steps concerned are highly interdependent and failure at any one point is very apt to make ineffective the care exercised in the operation of other parts of the process. Accordingly there is no factor involved which is so insignificant that it may be overlooked.

It is fortunately the case that, after long experience, there has been developed a more or less standard technique which can be applied in most instances. The advantage of having such a common method to resort to lies in the fact that it affords a basis of comparison between different materials prepared in the same manner. In many cases this is of the greatest value because variations in technical methods often lead to widely different results.

However, it is quite impossible to apply any method effectively without understanding the reason for each of its steps. Mere application of a method by rote, with the expectation that thereby an accurate or standard result will be achieved, is quite a wrong procedure. There are so many factors involved in the use of reagents with protoplasmic material that an appreciation of the relations set up in each case is highly desirable.

Often very serious inconveniences can be avoided by a clear understanding of just what is essential in the operation. To illustrate this point a very simple case may be instanced. Many beginners have great difficulty in making paraffin sections adhere to the glass slip.<sup>1</sup> Much valuable time is wasted in trying out various expedients when an understanding of the essential requirement of the process would immediately lead to proper practices.

There are only two elements concerned in this step which require consideration. In the first place, the glass has to be chemically clean, which may be easily tested by noting whether or not the dilute albumen water adheres as an even film over the entire surface. If this be accomplished the next consideration is to see that the paraffin ribbon is completely extended so that at all points it ultimately comes down into intimate contact with the glass surface. This can also be tested after the complete evaporation of the water by examining the section on the reverse side. If the ribbon has been completely spread each section is seen in its entirety applied closely to the surface of the glass. If it is not in contact with the glass an air space is clearly observable. Thus, by applying two simple tests, one may be assured before removing the paraffin that the sections are going to adhere.

Similarly, in every other step of the process, such definite requirements must be met and for most of these there are clear tests. An understanding of the factors involved in each case accordingly makes one certain of results.

For the beginner the one sure method of progress lies in a careful study of the errors that develop and their elimination through an understanding of the principles which have been violated. In the beginning, therefore, progress is merely the perpetration of a series of errors and their correction as indicated. In order that this desirable end may be reached it is best to keep very careful records of all processes, noting the time element, the concentration of reagents, the interrelations of the steps, the nature of external conditions, and the appearance of the material as it goes through the process. Cards on which these records may easily be made have been designed,<sup>2</sup> and it is suggested that the beginner either make use of these or that he prepare others suited to his own particular

<sup>1</sup> Confusion and uncertainty arise from the use of one term for two different objects and it is an unjustifiable practice. Accordingly, contrary to general practice, the glass upon which the sections are spread, usually  $1 \times 3$  inches in dimensions, will be called the "slip"; the term "slide" being restricted to the mounted preparation, consisting usually of the object surrounded by an appropriate medium and lying between the "slip" and "cover glass."

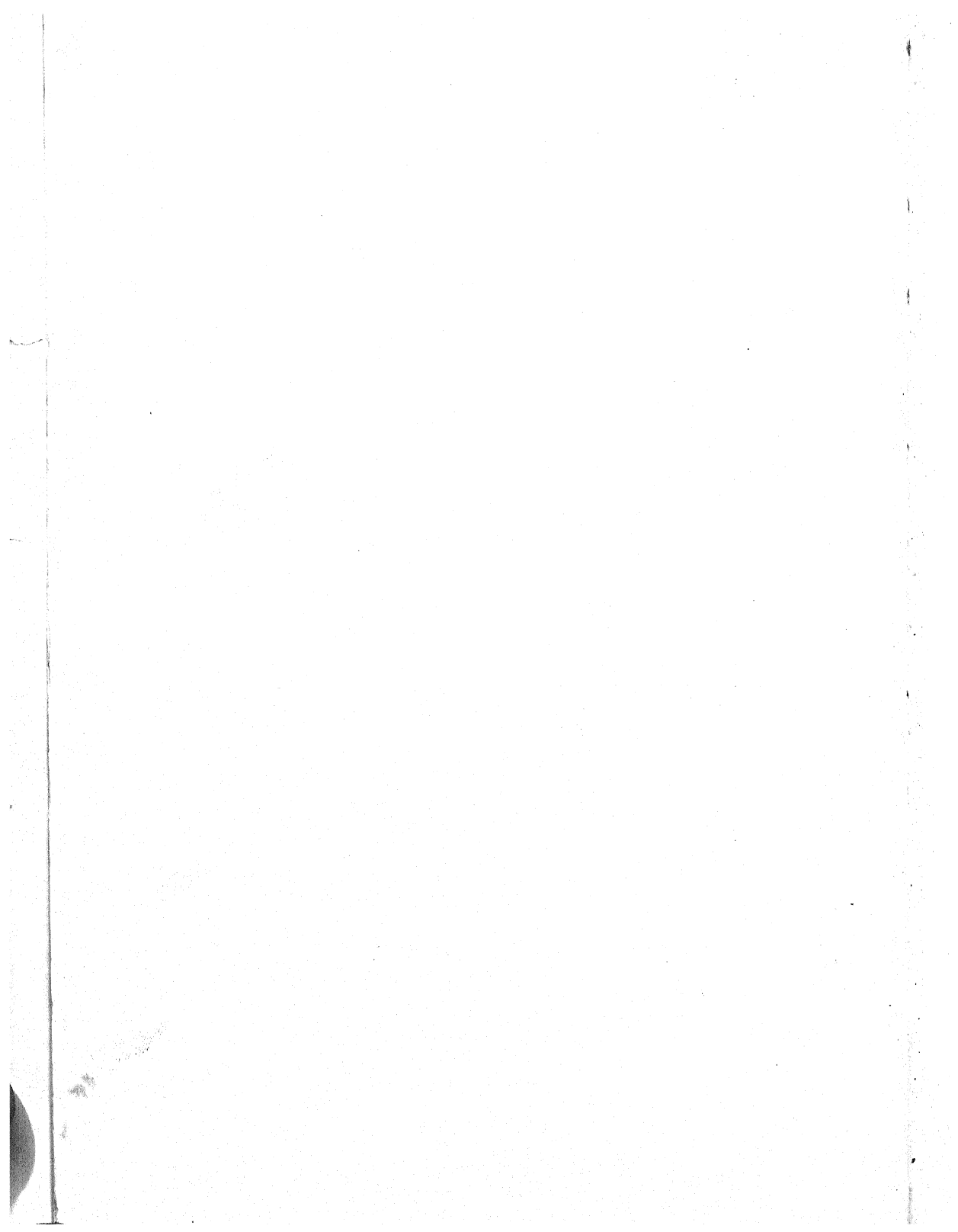
<sup>2</sup> Scammon, R. E. *Kan. Univ. Sci. Bull.*, 4:4, 1908.

Hance, R. T. *Trans. Am. Micr. Soc.*, 35:1, 1916.

requirements. At any rate, the necessity for accurate and complete records cannot be escaped.

The conditions to be met in the preparation of microscopical slides are set by the nature of the material to be studied and by the limitations of the instruments used for their study. In most cases the material is a mass of unstable colloidal substance, protoplasm, prone to rapid disintegration and of a size and character unfavorable for direct observation. It is generally necessary that this (1) be so treated as to undergo no essential structural change, but at the same time be so physically altered as to lend itself to manipulation; (2) that it be reduced to dimensions favorable to microscopical observation; (3) that the diverse elements present be colored differentially so as to become more strikingly apparent; (4) that the portions thus treated be mounted between pieces of glass of a convenient size and within a medium which will preserve them and present correct conditions of light refractions. These four requirements give rise to a series of processes, each of which is characterized by its own peculiar manipulations. They are described in their general application in Part I, and in their special uses in the succeeding chapters.





# CONTENTS

PREFACE TO THE SECOND EDITION . . . . .	v
PREFACE TO THE FIRST EDITION . . . . .	vii
INTRODUCTION . . . . .	ix
LIST OF CONTRIBUTORS . . . . .	xv

## PART I

### GENERAL METHODS

#### CHAPTER

I. Section and Non-section Methods of Preparing Microscopical Slides . . . . .	3
C. E. McClung	

## PART II

### SPECIAL METHODS

II. Methods for the Study of Fresh Material . . . . .	41
Microdissection . . . . .	43
Physical Agents:	
Free hand Manipulations . . . . .	Sven Horstadius 43
Free hand or Partly Mechanical Methods of Microinjection with wide Range of Control . . . . .	H. McE. Knower 51
Micrurgical Technique for the Study of Cellular Phenomena . . . . .	62
Robert Chambers, M. J. Kopac	
Chemical Agents:	
Vital Stains . . . . .	Nathan Chandler Foot 110
Supravital Stains . . . . .	Florence R. Sabin 117
III. Bacteriological Methods . . . . .	132
H. J. Conn, F. B. Mallory, Frederic Parker, Jr.	
IV. General Botanical Microtechnique . . . . .	William Randolph Taylor 155
Fats . . . . .	Sophia H. Eckerson 222
V. Cytological Methods . . . . .	246
C. E. McClung, Ezra Allen, R. T. Hance, J. W. McNabb, E. V. Cowdry	
VI. Embryological Methods . . . . .	C. E. McClung, Ezra Allen, Ruth McClung Jones 279
VII. Histological Methods:	
Methods of Studying Red Blood Cells . . . . .	Raphael Isaacs 287
Methods of Studying Leucocytes . . . . .	Ethel M. Slider, Hal Downey 324
Technique for the Study of Bone . . . . .	Paul G. Shipley 344
Technique for the Study of Dental Tissues . . . . .	353
H. R. Churchill, J. L. T. Appleton	

Methods for the Intercellular Substances of the Connective Tissues . . . .	402
F. B. Mallory, Frederic Parker, Jr.	
Methods for Preparing Muscle and Electric Tissues . . . .	Ulric Dahlgren 420
Neurological Technique . . . . .	W. H. F. Addison 437
Silver Methods for Boutons terminaux and Neurofibrils . . . . .	481
William C. Gibson	
Neuroglia and Microglia (The Metallic Methods) . . . . .	489
Wilder Penfield, William Cone	
VIII. Protozoological Methods . . . . .	D. H. Wenrich 522
IX. Fixation and Fixatives . . . . .	C. E. McClung, Ezra Allen 552
X. Stains and Staining . . . . .	C. E. McClung 573
Aniline Dyes . . . . .	H. J. Conn 577
XI. Miscellaneous . . . . .	615
C. E. McClung, C. H. Heuser, H. McE. Knower, M. H. Knisely,	
G. H. Scott, Ethel B. Harvey, C. J. Sutro	
INDEX . . . . .	675

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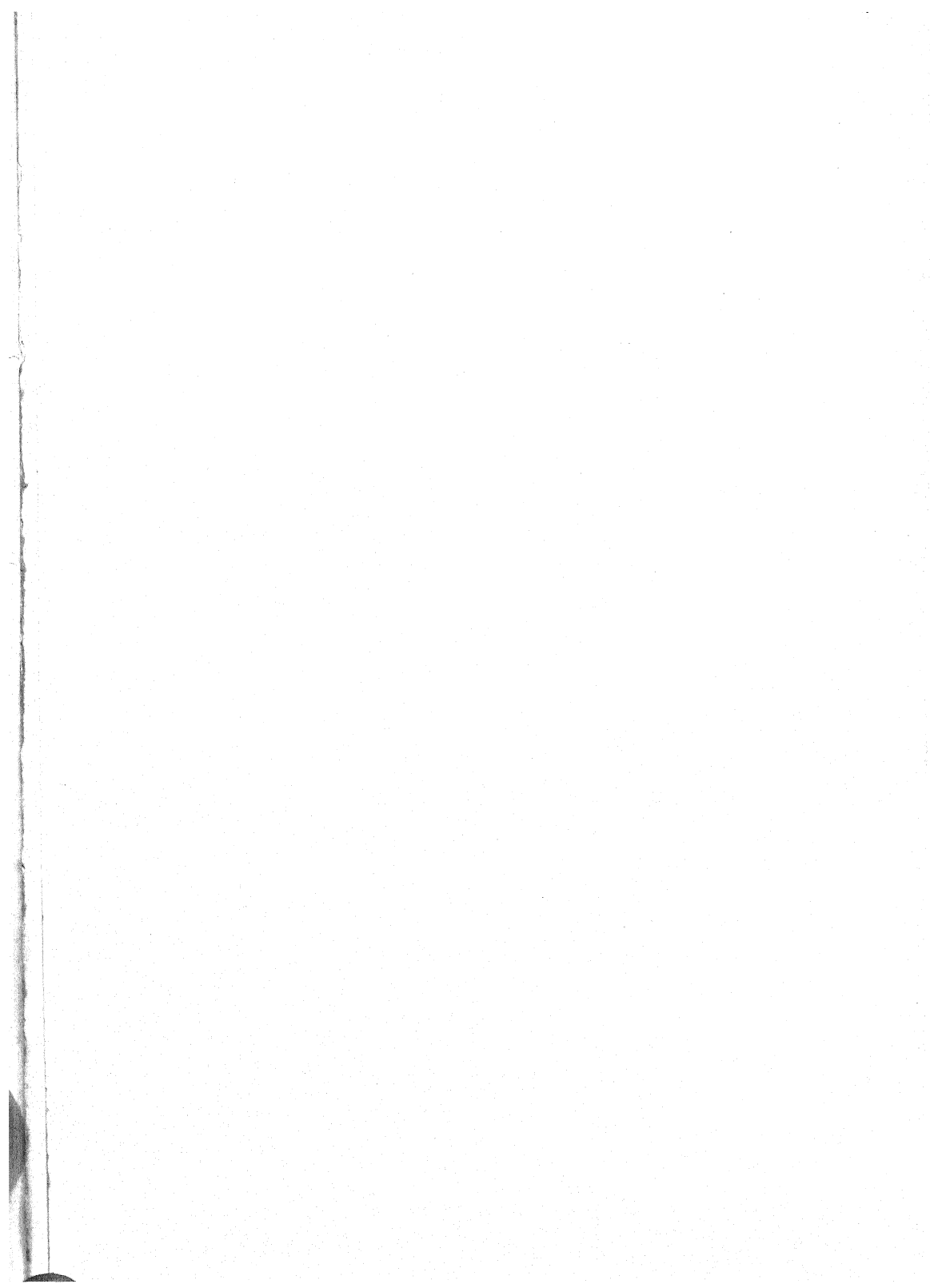
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**PART I**  
**GENERAL METHODS**





## CHAPTER I

### SECTION AND NON-SECTION METHODS OF PREPARING MICROSCOPICAL SLIDES

C. E. McCLUNG

METHODS OF REDUCING MATERIAL TO A PHYSICAL STATE ADAPTED TO MICROSCOPICAL OBSERVATION 3. Section Methods 3. Non-section 5. DETAILED DESCRIPTION OF METHODS 6. Paraffin 6. Collodion 29. Freezing 32. Smear 33. Stretching 35. Teasing 36. Grinding 37. Macerating 37. Recent Modifications of the Paraffin Method 38. A New Mounting Medium 40. Sectioning Refractory Tissues in Paraffin 40.

#### A. METHODS OF REDUCING MATERIAL TO A PHYSICAL STATE ADAPTED TO MICROSCOPICAL OBSERVATION

It is only rarely that material is naturally in a condition to be examined under the microscope without some manipulation. In general it is necessary to so reduce the mass as to permit light to pass through the object, since this is the manner in which most microscopical observation is conducted. There are two general means by which this requirement may be satisfied; first, the section method, which is the cutting of the mass of material into thin sections usually by means of a knife so that light may pass through; and, second, non-section methods, whereby the mass of material is separated by some physical or chemical means into sufficiently small particles so that light may pass through and around them. Of these, the method of sectioning is the one most commonly employed.

#### I. Section Methods

The modern methods of cutting sections represent a gradual development from the earlier simple ones up to the present highly specialized and more complicated procedures in common use. The older ones still find limited application in some cases.

1. **Unsupported Material.** Naturally the first means employed was that of simply cutting sufficiently thin slices from the fresh material by means of a razor. This is now restricted largely to hasty examinations of plant tissues. (For a description of the method see p. 176).

2. **Imbedded Material.** It was soon found necessary to support the mass of tissue to be cut in some way, which was done by some form of imbedding. At first a support, such as would be afforded by pith or cork, was employed, but later the mass of material was included in melted paraffin or wax. These procedures, however, merely support the exterior

of the tissue and in no wise contribute to the rigidity of the interior of the mass. They therefore are rarely used.

**3. Infiltrated Material.** Methods of infiltration were then developed. In these the specimen is so treated that the supporting material may enter intimately into the mass and give it support at every point. Only by the use of such means has it become possible to secure serial sections of the uniform thickness required by modern microscopical methods of study. There are two media which are commonly employed for infiltrating, paraffin and collodion.<sup>1</sup> These have each their advantages and disadvantages and the method chosen must have regard to the character of the material and the purpose for which it is to be used. The details of application of these two substances for infiltration will be found under appropriate headings, but it may be desirable to indicate here the chief characteristics of the two.

*Paraffin as a Medium.* The advantages of paraffin are that it readily permits one to secure thin sections preserved in a fixed serial order. The disadvantages are that large paraffin sections cannot readily be obtained and that some shrinkage occurs from the action of the clearing and infiltrating media, especially at the temperature required for keeping paraffin melted.

*Collodion as a Medium.* The advantages of the collodion method are that large sections may readily be cut and in the absence of heat at any stage of infiltration shrinkage is avoided. Collodion may also be indicated for certain tissues which become hard when paraffin is employed for infiltration. It is also true that, following certain fixatives, collodion may preserve the finest cytological details in cells which by paraffin infiltration become seriously altered. It is therefore desirable in every case to try both the paraffin and collodion methods if the very best results are desired. The disadvantages of collodion are that thin sections cannot readily be cut and the process of sectioning is relatively slow and in some ways more difficult, especially if serial sections are desired.

*Other Media.* For special purposes gelatin, soap and other media may be better adapted than the two commonly used. (For an account of some such cases, see p. 178).

**4. Choice of Medium.** As is indicated, the choice of the infiltrating medium must depend on the nature of the material and the purpose for which the sections are cut. By considering the advantages and disadvantages of the various media it should be possible to select one which will be most suitable.

**5. Grinding.** This method is applicable only to such dense substances as bone or teeth and therefore has a very limited use.

<sup>1</sup> Collodion, celloidin, parlodion, etc.

**6. Characteristics of Section Methods.** The advantages of sections as a means of microscopical analysis of structure are that the elements are preserved in their normal relations to each other and that they are in so thin a layer that the intimate details of cell and tissue structure may be observed. The disadvantages lie in the fact that in thus reducing the mass to thin sections, elements are cut through regardless of their natural boundaries and fragments of cells are then presented for study at certain points.

No absolute rule can be given with regard to the proper thickness to which sections should be cut to secure best results. It all depends on the purpose in view and on the character of the material. In general, it may be said that, for optimum staining, the cells should be cut through, that is the sections should not exceed the average diameter of the cell, but it is also the general rule that for the study of very fine details of structure, thinner sections are best. The reason for this is that differentiation of stains, especially the iron hematoxylin stain, can best be secured in the absence of any great variation in the character of the material in the section. Thin sections reduce this variation. On the other hand when it is desired to study general relations, thick as well as thin sections are necessary. In chromosome studies, where smear preparations are not convenient, thick sections will preserve the entire complex in certain cells so that there is no danger of counting fragments. Very thin sections range from 1 to  $5\mu$ , medium sections from 5 to  $10\mu$ , and thick sections from  $10\mu$  up to 20 or  $30\mu$ . Sections of average thickness will be found generally most convenient for study.

## II. Non-section Methods

If material is prepared by non-section methods the choice of means is strictly limited by the physical characteristics of the tissue. There is, therefore, less flexibility here than in the case of section methods which can be applied to almost any type of tissue.

**1. Smearing.** Of the non-section methods, the one most commonly employed is known as the smear method. The use of this is restricted to tissues of a fluid or semi-fluid character which may be spread in a thin film upon a glass surface. Such tissues as blood lend themselves most readily to this operation and in this particular case almost require the application of such a method.

**2. Stretching.** This method is rigidly limited in its application, being useful in the case of natural membranes which may be extended in so thin a layer that they are observable like sections. The only require-

ment here is a means for holding the membrane stretched while it is fixed. After that it is treated like a section.

**3. Teasing.** The operation of teasing can be applied well only to fibrous tissues such as tendon and muscle. By means of needles the connective tissue is combed away from the fibers leaving them individually free or collected into small groups.

**4. Macerating.** The three previously mentioned methods are all physical in character while maceration is a chemical method of dissociating elements. In its use a weak fixative is employed which differentially affects the various tissues so that the connective tissue becomes soluble and leaves free the elements which it bound together. This method may be used with a wide variety of materials.

**5. Choice of a Non-section Method.** The non-section methods are limited in their use by the physical characteristics of the tissue and so the selection of any one of them is definitely indicated and does not require further discussion.

**6. Characteristics of Non-section Methods.** As contrasted with section methods, the non-section methods, aside from stretching, present individual elements entire, but dissociated from their normal relations. It is obvious, therefore, that when possible it is desirable to use both types of procedure.

## B. DETAILED DESCRIPTION OF METHODS

### I. Paraffin Method

Since this is the method most commonly employed, the complete series of processes involved will be stated in detail and this will serve as a basis upon which to consider other less used methods. These steps follow in order, are mutually interdependent, and so have to be considered as a whole although it is desirable to separate the entire account into its steps in order that these may be better presented.

While the general directions supply the information necessary for the accomplishment of certain results with a given method, there are so many details involved that a definite statement of the application of the paraffin method to a particular piece of tissue will be given. Because so often the technique is applied to visceral organs, one of these, the liver, will be used as an example.

**1. Killing** is the act of stopping the vital processes of an organism. It might seem to be a relatively unimportant detail but it turns out to be quite the contrary, for in some cases, at least, the entire appearance of the cell depends upon this step. For instance, in the case of the first

spermatocytes of the Orthoptera, if the animal is killed by an anesthetic, the cytoplasm has a fine fibrous character in the metaphases and the chromosomes are well spread and clear in outline. On the contrary, if another animal is treated with cyanide, the cytoplasm at a corresponding stage is coarsely granular, the spindle is not over half the length of that in the other case, and the chromosomes are closely drawn together and indistinct in outline. Since these are elements of the greatest significance in the structure of the germ cells, it is quite apparent that the method of killing is of the utmost importance. In many cases this may not be so significant a step, but it is something which should always be considered. In general, the use of an anesthetic is recommended, particularly in the case of smaller animals. Larger animals may be killed by a blow on the head or by severing the spinal cord. In every instance the operation should be completed as rapidly as possible.

If, for example, a rat is used, the animal should be placed in a glass cylinder with a tight fitting cover. Drop into this a piece of absorbent cotton saturated with chloroform. Allow the animal to remain until unconscious and then remove it from the vessel. Open the abdominal cavity by a median ventral incision if visceral organs are desired. From the organ cut a segment which should be about 5 mm. in linear dimensions. If it is desired to carry through any other pieces of tissue they should be removed at the same time.

**2. Marking.** This is a means of attaching a clear and unequivocal designating number or character to a specimen. As soon as the material to be processed is removed from the body of the animal it should be definitely marked in some way so that it may not be mistaken for any other specimen. In many instances it is important to preserve the animal from which the material has been removed, and in this case the same designating character should be placed on the animal and upon the part removed from it. In case any considerable number of specimens are being investigated, the simplest means is to mark each with a serial number so that there may be no duplications. Such lot numbers are conveniently entered in a book, just as are the accessions in a library. Later, for purposes of convenience, classifications according to the subject matter can be made, but the existence of one unequivocal designation for each specimen in the collection is of the greatest importance.

It is sometimes difficult to carry through the designating mark with the material, but it may be done by employing a vessel for each specimen. A very convenient way when many specimens are to be prepared at the same time, is to put the lot number on a convenient sized piece of paper with a pencil or water-proof India ink, and on the reverse side of the paper the fresh material just removed from the organism. Then the two are immediately immersed in the fixing fluid. In this way the piece of

ment here is a means for holding the membrane stretched while it is fixed. After that it is treated like a section.

**3. Teasing.** The operation of teasing can be applied well only to fibrous tissues such as tendon and muscle. By means of needles the connective tissue is combed away from the fibers leaving them individually free or collected into small groups.

**4. Macerating.** The three previously mentioned methods are all physical in character while maceration is a chemical method of dissociating elements. In its use a weak fixative is employed which differentially affects the various tissues so that the connective tissue becomes soluble and leaves free the elements which it bound together. This method may be used with a wide variety of materials.

**5. Choice of a Non-section Method.** The non-section methods are limited in their use by the physical characteristics of the tissue and so the selection of any one of them is definitely indicated and does not require further discussion.

**6. Characteristics of Non-section Methods.** As contrasted with section methods, the non-section methods, aside from stretching, present individual elements entire, but dissociated from their normal relations. It is obvious, therefore, that when possible it is desirable to use both types of procedure.

## B. DETAILED DESCRIPTION OF METHODS

### I. Paraffin Method

Since this is the method most commonly employed, the complete series of processes involved will be stated in detail and this will serve as a basis upon which to consider other less used methods. These steps follow in order, are mutually interdependent, and so have to be considered as a whole although it is desirable to separate the entire account into its steps in order that these may be better presented.

While the general directions supply the information necessary for the accomplishment of certain results with a given method, there are so many details involved that a definite statement of the application of the paraffin method to a particular piece of tissue will be given. Because so often the technique is applied to visceral organs, one of these, the liver, will be used as an example.

**1. Killing** is the act of stopping the vital processes of an organism. It might seem to be a relatively unimportant detail but it turns out to be quite the contrary, for in some cases, at least, the entire appearance of the cell depends upon this step. For instance, in the case of the first

spermatocytes of the Orthoptera, if the animal is killed by an anesthetic, the cytoplasm has a fine fibrous character in the metaphases and the chromosomes are well spread and clear in outline. On the contrary, if another animal is treated with cyanide, the cytoplasm at a corresponding stage is coarsely granular, the spindle is not over half the length of that in the other case, and the chromosomes are closely drawn together and indistinct in outline. Since these are elements of the greatest significance in the structure of the germ cells, it is quite apparent that the method of killing is of the utmost importance. In many cases this may not be so significant a step, but it is something which should always be considered. In general, the use of an anesthetic is recommended, particularly in the case of smaller animals. Larger animals may be killed by a blow on the head or by severing the spinal cord. In every instance the operation should be completed as rapidly as possible.

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paper is firmly attached to the specimen by coagulation and may then be carried through the subsequent processes without danger of dislocation. If material, such as that from plants, will not adhere, it may be made to do so by using a little Mayer's albumen. If record cards are employed, the lot number is placed upon the proper card and, as the material passes through the subsequent steps, these are carefully recorded in order. Danger of neglecting to keep records of essential steps is thus avoided. The card, when completed, forms a part of the permanent record relating to the specimen and may be consulted at any time.

3. **Fixing** is the process of preserving, by means of coagulation, the normal structural characters of organs, tissues and cells. Before fixation the greatest care must be exercised in manipulating tissues. They are easily injured by pressure and in many cases it is best to fix *in situ*. Where this is not possible the operation of removal should be conducted with the greatest care and the tissue disturbed as little as possible. In general, the smallest piece of tissue that will suffice should be used because the fixative penetrates larger pieces with difficulty. As noted under different fixatives, the degree of penetration varies. Picro-formol-acetic combinations penetrate readily, as does the acetic acid, alcohol and chloroform combination, but osmic mixtures have very slight power of penetration. On the average, pieces of tissue from 2 mm. to 8 mm. in diameter are best for all purposes.

The process of fixation is one of the most important in the series. Upon its character all the remaining steps and the ultimate result depend. The endeavor here is to preserve in a permanent form, as nearly as may be, the exact configuration of cells and tissues in the living state. Obviously this cannot be exactly accomplished because living protoplasm is a gelatinous or semi-fluid material, and after fixation it becomes a solid. Since, however, this change is accomplished by the process of coagulation it is possible to preserve in the solid form essentially the same relations that obtained in the living state. It is only after considerable experience that one may accurately judge the operation of fixation, but in general it may be said that any evidence of shrinkage or swelling indicates that the process has not been perfect in its operation.

It is claimed by some that, by the very nature of fixation, the normal structure has been destroyed, but a careful comparison between living cells and similar cells after fixation has demonstrated that the picture of the preserved material accurately represents that existing in life. This is a matter of fundamental importance, for, unless we are studying normal relations in the preserved material, nothing is to be gained by its use. It may be said, however, with every assurance, that a properly prepared specimen affords an accurate means for determining normal structural

conditions. Of course it is necessary to interpret microscopical images and, unfortunately, by speaking in terms of solid substances in cells, a misinterpretation is involved. It is customary to refer to certain aggregates in cells as "granules" when, as a matter of fact, in the living cell these are droplets of varying degrees of density. If it is understood what is meant by such terms, however, there is no danger of misconception.

To be effective fixation must be accomplished as rapidly as possible, before any post mortem changes have taken place in the tissues. Therefore it is often desirable to apply the fixing fluid directly to the part in the body of the animal. This can easily be done in small specimens by opening the body, and in large ones by means of injections. There are very few instances in which a delay in applying the fixative is desirable. The general rule is to fix immediately and avoid any change due either to temperature or evaporation.

*Reagents.* The reagents chosen should be adapted to the purpose for which the material is used. Some fixatives preserve nuclear conditions especially well, others those of the cytoplasm. In this general description of processes it will be assumed that the nuclear conditions are primarily in mind. The particular fixative that may best be used, again, depends upon the nature of the material. In some cases this requires a reagent which penetrates with extreme vigor and rapidity. In others it is necessary to choose one which is slower and more delicate in its operation. What is required in each case can be determined only by experience. In the following description it will be assumed that the material considered is of the character found in some visceral organ in which extreme difficulties in penetration will not be met. Under these circumstances extended recent experience has indicated that one of the picro-formol-acetic mixtures may be selected with assurance of getting at least a good fixation in almost every instance. In most cases it is the best that can be obtained.

The three substances, picric acid, acetic acid, and formalin have individually different effects upon protoplasm. Thus picric acid alone shrinks it while both acetic acid and formalin have a swelling action. The proportions of these reagents must therefore be adapted to the particular kind of material treated. Only previous experience, or a general knowledge of the nature of the material, will make it possible to choose at first the particular combination which will be most effective. Commonly, however, the fixation obtained with Bouin's original formula will be good (p. 560). In case very resistant tissues are involved, the two fluids invented by Carnoy (p. 558) will commonly be most effective.

In recent years it has been found that certain non-coagulating substances like urea and sugar, when added to fixing fluids, have specific results. The choice here again depends upon the effect desired, which

can be learned only by experiment. However, in general it appears that the addition of something like 2 per cent of urea to certain fixing fluids materially improves the character of their operation. In the case of plant cells various sugars show specific effects (p. 207).

Assuming that the specimen has been appropriately excised and marked and that the proper fixing fluid has been prepared, the next step is to apply the fixative under appropriate physical conditions and for the proper length of time. Ordinarily the fixative may be used at room temperature, but for certain results, higher or lower temperatures may be necessary. It has been found, for instance, that adding urea to Flemming's fluid and fixing Orthopteran cells at a temperature of 0°C. reverses the relative density of nucleus and cytoplasm as compared with the results of ordinary fixation—that is, the nucleus appears as a very dense body. For further discussion of this topic see Flemming's fluid and Allen's fluid (p. 561). The length of time required for the best fixation depends, among other things, upon the nature of the material, the character of the fixative and the temperature. Some fixatives like the osmic acid mixtures over-fix if employed for too long a period, while others, like certain picro-formol-acetic combinations, may be allowed to act indefinitely without injury. As a general guide it may be said that for ordinary tissues in pieces not over 4 mm. in diameter, fixation will be accomplished in from two to twenty-four hours. In view of the flexibility of the P.F.A. mixtures it is desirable in the beginning to employ them because the length of their operation has very little influence on the character of the end result. That is one of their many advantages.

Since the reagents involved in these fixations are vigorous chemicals, it is generally necessary to use glass or porcelain containers and to avoid bringing any metal instruments into the fluids. If the specimen has been fastened to a piece of paper with the designating number upon it, it is convenient to float the material on the surface of the fixing fluid where it will be held by surface tension. This is an advantageous position for the material because it always remains at a normal concentration of the fixative, whereas if it lies at the bottom of the vessel in a limited amount of fluid, the water extracted from the material dilutes the fixative as time goes on. To avoid any such contingency it is well to use a sufficient quantity of the fixative so that additional water will not affect its concentration. At the end of the process of fixation the material is completely saturated with the reagent and a certain proportion has chemically combined with the protoplasm.

*Hardening.* The stronger fixatives, like the chromic acid combinations, mercuric chloride mixtures, etc., harden sufficiently so that no subsequent treatment is required. In the case of some of the more delicate

fixatives, however, further treatment with alcohol of high concentration or with bichromate of potash is required. The material should be kept in 95 per cent alcohol or in 5 per cent bichromate until the desired consistency is reached, a period varying considerably with the size and character of the specimen. There is always a danger of excessive hardening when treating unfamiliar material, but this can be avoided by testing at intervals. Due consideration should be given to the question of whether sectioning is to be done in paraffin or collodion.

4. **Washing** is the process of removing the excess fixative from the specimen. The medium to be employed depends upon the nature of the fixative, but commonly it is water following aqueous fixatives. Only in the case of fixations with such weak agents as picric acid in the absence of formalin, is it necessary to wash out with alcohol in order to avoid maceration. Washing is completed when approximately all of the uncombined fixative is removed from the tissue. It is sometimes difficult to determine when this has been accomplished, but with the picro-formol-acetic mixtures it can be assured when the yellow color of the picric acid is no longer strongly manifest in the washing fluid. As a general rule, for fixatives like Flemming, Hermann (p. 560), etc., it is desirable to wash out at least as long as the time involved in fixation and to be on the safe side it should in most cases be longer than this period, if not to exceed twenty-four hours. It is possible to facilitate the removal of the fixative in some cases by adding certain substances to the washing fluid. Thus with picric acid, the presence of a small quantity of lithium carbonate lessens the washing period, while with mercuric chloride, iodine similarly hastens the process. It must be remembered also that in subsequent steps a continuation of the process of extraction may be counted upon. In most cases, moreover, a small quantity of the fixative will do no damage up to the time of staining. This is especially true of the P.F.A. mixtures. On the other hand, when mercuric chloride has been used, complete removal is necessary, otherwise the crystals remaining produce artifacts and also injure the cutting edge of the microtome knife. If washing has been incomplete, in cases where chromic acid has formed a part of the mixture, the staining reaction with hematoxylin may be reversed in the nucleus. The general rule, accordingly, which should be adhered to, is to remove as completely as possible all traces of the fixing agents.

To carry out the process of washing, in the example selected, the tissue is removed to a tall Stender dish filled with water, where it may again be floated on the surface. Only the excess of the fixing fluid is removed in the water, which may be done in about an hour. After this it is transferred to vessels containing successively 30 per cent, 50 per cent and 70 per cent alcohol. The intervals in

these various strengths of alcohol may be, for the first two, an hour each, and for the 70 per cent a sufficient length of time to remove most of the fixative.

**5. Staining in Toto.** At this point it is sometimes advantageous to stain the mass of tissue entire, or, as it is commonly designated, in toto. Not all staining agents are useful for this purpose. Amongst the best are the carmine and cochineal mixtures. Hematoxylin combinations are less effective and rarely can aniline stains be employed at all. One of the best stains for this purpose is the alum-cochineal mixture, especially for embryos (pp. 284, 612). Obviously, in this process, it would be necessary to employ a reagent which does not over-stain. Where material can be treated in toto, it is, of course, a very great advantage because the sections are all ready for mounting as soon as the cutting and attaching to the slip are accomplished. For embryological purposes this is a very good and frequently used method, but in cytological work it finds only limited application.

To stain the piece of liver, place it, after dehydration, in alum-cochineal, diluted one-half, for five to ten hours. This gives a nuclear stain and if the picric acid has not been completely washed out a double stain will be obtained in which the yellow of the picric acid contrasts fairly well with the red of the cochineal. After staining, wash the specimen for thirty minutes in water to remove the contained alum which otherwise would crystallize in the alcohols and cause difficulty in sectioning. A very precise and delicate stain of great transparency can be obtained by decolorizing with acid alcohol.

**6. Dehydration** is the process of replacing water in the specimen, usually with alcohol. In most cases the material, up to this point, has been in aqueous solutions, but it now becomes necessary to remove the water. It is not a matter of indifference how this is done, for the attraction between water and alcohol is so violent that, in mixing, diffusion currents or osmotic pressure often severely damages the tissues. For this reason it is customary to proceed gradually in the removal of water in either one of two ways: (1) by passing the material through ascending grades of alcohol of 30 per cent, 50 per cent, 70 per cent, 83 per cent and 95 per cent, and sometimes up into absolute alcohol; (2) increasing the concentration of the alcohol gradually by adding it slowly in small quantities to the water in which the specimen is placed. Whatever method is employed for adding the alcohols, arrangements should be made to secure a mixing of the fluids, since the lighter alcohol remains on the surface. A convenient way of doing this is by means of capillary syphons which add the alcohol to the water slowly, a drop at a time. In this case, facilitate mixing by agitation, either by stirring devices or by the

passage of a stream of air bubbles through the mixture. A vessel charged with compressed air may be connected with a small glass nozzle which reaches to the bottom of the fluid, and by regulating the flow of air the fluids may be kept completely mixed. It is advantageous to draw off portions of the mixture at intervals thus increasing the concentration of the alcohol (p. 254).

Still another way is to suspend the specimen, in a small quantity of water in a vessel with a membranous bottom in a tall cylinder of 95 per cent alcohol. The water, because of its greater specific gravity, descends to the bottom of the cylinder, leaving the specimen bathed in the stronger alcohol at the top. This is not so satisfactory because the interchange between the water and alcohol may be violent.

At the end of dehydration the water is completely removed from the specimen and in its place is found absolute alcohol, if this be used. It is very important to see that this end is reached, and sufficient time should be given in the final strength to secure this result. There is little danger of shrinkage in 95 per cent alcohol, and for this reason it is a safe practice to permit the tissue to remain in it for twenty-four hours or longer.

The various grades of alcohol have their particular effects upon the material and some of these have practical advantages. In lower grades maceration may be accomplished, if this is desired, while in the intermediate, such as 70 per cent, material may be kept indefinitely. Beyond the strength of 70 per cent, especially in absolute alcohol, hardening occurs, accompanied by shrinkage, and for this reason it is not desirable to prolong action here any more than is necessary.

While it is possible to proceed directly from 95 per cent alcohol or absolute, into xylol, it will be found a matter of convenience to pass the material into a mixture of equal parts of 95 per cent alcohol and xylol. This mixture should be entirely clear and if it is not so it indicates that the alcohol has fallen below 95 per cent and sufficient absolute should be added to make a clear mixture. It is sometimes necessary, in the case of delicate tissues, to replace the alcohol very gradually by the drop method.

**7. Clearing** is the process of removing the alcohol in the specimen with some fluid miscible with paraffin. In order to carry the material from alcohol into paraffin it is necessary to interpose this intermediate step. For this purpose some substance must be used which, on the one hand, mixes readily with alcohol and, on the other, dissolves paraffin—commonly one of the hydrocarbons, such as benzol, xylol or toluol. Chloroform, turpentine or any of the essential oils can be utilized, and for many purposes aniline oil is advantageous. If the essential oils are used the piece of tissue becomes entirely translucent when the alcohol is completely removed, hence the name of the process. That is true also of

aniline oil, but xylol or chloroform does not produce so marked a clearing effect and it is therefore more difficult to determine when their action is complete.

Because it is sometimes difficult to secure full dehydration within a limited time, the practice of clearing in aniline oil has been growing. If aniline is employed the tissue may be transferred to it from 95 per cent alcohol or even from a lower grade (p. 256). To avoid carrying the aniline over into the paraffin, treatment with chloroform is given the tissue until most of the aniline is removed. Two changes of five minutes each are often sufficient.

In clearing reduce the time of the operation to the shortest period since shrinkage occurs. In most cases, change the clearing fluid at least once in order to avoid carrying over any alcohol into the paraffin. At the end of this process the alcohol is completely removed and all portions of the tissue are filled with the clearing agent. A piece of tissue, such as is being described, would probably require two or three hours for complete removal of the alcohol.

8. *Infiltrating* is the process of replacing the clearing agent in the specimen with melted paraffin. Paraffin at ordinary temperatures is a solid and in order that it may penetrate the tissues and replace the clearing agent it is, of course, necessary to make it a fluid by means of heat. This has introduced a complication of considerable magnitude, which, however, may be avoided by a very simple means. It was formerly customary to employ very elaborate water baths with thermo-regulators in order to keep the melted paraffin at a constant temperature. The same end may be readily accomplished in a much simpler and more effective fashion by placing the paraffin in a cylindrical vessel, like a tumbler, over which is suspended an incandescent lamp which will give sufficient heat to melt the upper portion of the paraffin. The 150 watt nitrogen filled bulbs on the market now do this very well. In practice, the tumbler, three-fourths full of paraffin, is placed beneath the incandescent bulb which is so adjusted in relation to it that a layer approximately an inch in depth remains in a fluid condition. Into this is transferred the specimen saturated with clearing fluid. Some materials require a gradual substitution of the clearing agent, which is accomplished by dissolving increasing amounts of paraffin in the bath of clearing fluid in which the specimen lies.

Under the conditions of the operation just described the piece of tissue drops to the bottom of the melted paraffin and comes to rest in a layer near the melting point. If the temperature increases, the paraffin melts down further, but the specimen drops with it and so it never can become overheated. At the same time the clearing fluid, being lighter than the

paraffin, rises to the surface, where the temperature is greatest and is thus more rapidly evaporated. The piece of tissue remains in the melted paraffin in general somewhat longer than the time required to clear the specimen. There is no way of telling when the clearing fluid is completely removed by the appearance of the material, and so some such arbitrary rule has to be followed. While immersed in melted paraffin the material constantly undergoes shrinkage after most clearing fluids. The time of its operation is therefore reduced to a minimum.

There are many grades of paraffin determined by the temperatures at which they melt, and the choice of the particular one to be employed depends upon the character of the material and also upon the temperature at which the sections are to be cut. The general principle involved here is to select a grade of paraffin which, at room temperature, somewhat nearly approximates in density the specimen itself. If there is a wide difference in this respect the sections will be imperfect through compression, if the paraffin be too soft; or through cracking and breaking if it be too hard. Sections in soft paraffin form ribbons well, but are difficult to handle. Very hard paraffin may fail to ribbon except at high room temperatures. A marked difference in density between object and paraffin results in the generation of troublesome static electricity in sectioning. Under ordinary conditions a grade of paraffin melting between 54°C. and 60°C. is most useful, but in case very hard material is to be cut, a paraffin of higher melting point is necessary. Here enters another complication, however, because if a hard paraffin is cut at a temperature widely removed from its melting point it becomes brittle and does not section well. Under these circumstances it is necessary to raise the temperature of the room in which the cutting occurs in order to bring about a proper relation between room temperature and density of the paraffin (p. 620). For ordinary room temperatures the grade of paraffin chosen for specimens, such as a piece of liver tissue, should be of a melting point of about 58°C. The specimen would probably be completely infiltrated in three hours. If many specimens are being carried through at one time, transfer them into a fresh bath of paraffin. To completely evaporate the dissolved clearing agent allow the paraffin to remain in the melted condition for some time after the specimens are removed. Occasionally the jar of paraffin should be melted completely so that any suspended particles may sink to the bottom. No attention to the tissue is required while in the paraffin and other work may be carried on advantageously during this interval. By a careful arrangement of the different operations much time may be saved in this way.

At the end of the operation of infiltration the clearing fluid has been



completely removed and all interstices of the specimen are filled with paraffin in the melted condition.

9. **Imbedding** is the process of enclosing the specimen in a convenient block of solid paraffin. The size of the block is determined in a variety of ways, such as forming paper boxes of appropriate dimensions or by manipulating metal blocks upon a glass plate so as to produce a chamber of proper size. Perhaps the best imbedding method of all is the one called the watch glass method. In this an ordinary rounded watch glass is very lightly coated with glycerin by rubbing a moistened finger over the concave surface. Into the glass, by means of a warm pipette, is placed a quantity of melted paraffin from the dish in which the specimen has been infiltrated. With proper manipulation a rounded body of paraffin may be built up by cooling the outer surface and slowly adding at the center additional amounts of melted paraffin. At the same time a thin film of hardened paraffin forms at the bottom of the dish and when the conditions are right the specimen is removed from the infiltration vessel by means of warmed forceps and deposited in the proper position within the mass of melted paraffin in the watch glass. Here it is oriented in relation to the table surface so that the plane of sectioning is thus indicated. Care should be taken to see that the amount of paraffin above the specimen is of some extent because on cooling there is considerable shrinkage, and the specimen should lie completely enclosed within the hardened mass of paraffin. By conducting these manipulations within the area heated by the incandescent bulb, convenient conditions of temperature may be found for good imbedding.

As soon as the tissue is thus arranged, by blowing on the surface of the paraffin a film can be produced and when this is of sufficient thickness so that the water will not easily break it, the watch glass with its contents is lowered slowly into a dish of cold water. When it is completely submerged the glass is allowed to drop to the bottom of the container. The purpose of this rapid cooling is to harden the paraffin before it has time to crystallize.

One advantage of the watch glass method over others is that in this process of cooling the mass of paraffin shrinks towards the center and is brought into most intimate contact with the material. If, on the contrary, the paraffin may adhere to the rigid walls of a vessel on cooling, it tends to draw away from the specimen and thus produces a difference in density in the immediate vicinity of the specimen.

After a time the glycerin film between the paraffin and the glass dissolves and the block of paraffin rises to the surface. If the specimen has been carried through upon a slip of paper with a lot number upon it, this can be read through the thinner layer of paraffin at the bottom and the

material easily identified. Specimens thus imbedded may be kept indefinitely without change and cut at any time.

10. **Blocking and Trimming** are processes concerned with getting the specimen into a determinate block of paraffin which is of such a character as to produce readily a continuous series of sections when cut on the microtome. In preparing the material for attaching to the microtome, mount the block of paraffin with the contained specimen on some support which fits into the object holder. Most microtomes have metal discs that are designed for this purpose, but it is more convenient in general to use a cylindrical wooden block upon the end of which the paraffin mass is attached. A very convenient way to make this connection is to stand the wooden cylinder on end, after having first filled its pores with melted paraffin. A section lifter is warmed in a flame and laid upon the paraffined end of the wooden block and at once the paraffin mass is brought down upon this heated section lifter, which is then immediately withdrawn, thus bringing the paraffin and wooden blocks into contact. In this way the melted paraffin attaches the specimen firmly to the wooden support. Since the specimen was so adjusted that the plane of sectioning corresponds to the bottom of the mass of paraffin, the plane of cutting is now indicated by the upper surface of the wood. To bring about a firmer union between the paraffin and the wooden support, immediately after withdrawing the heated section lifter, plunge the mount into a vessel of cold water.

As a further preparation for sectioning, the excess paraffin about the specimen must now be removed. The manner of accomplishing this is as follows:

By means of a safety razor blade or similar cutting instrument, the paraffin is gradually trimmed up towards the specimen until there is produced a plane figure of four sides, two of which are parallel, the third at right angles to these two, and the fourth at a slightly acute angle with one of the parallel sides; in other words, a trapezoid with the specimen lying near the center. It is necessary to have two parallel sides, which on sectioning are the ones in contact with the cutting edge of the knife. The purpose of the inclined edge is to indicate one side of the ribbon by means of a notched contour. This is very helpful later in handling the segments of the ribbon.

In trimming the block, two points should be borne in mind aside from those already mentioned. First, do not extend the cutting below the level of the bottom of the material very far. The reason for this is that it is best to have as much support as can be gained, and, by removing no more paraffin than is necessary to clear the material, this is aided. Sometimes, when a very long piece of substance is being cut, it is desirable to trim only part way down its length and then by successive trimmings to carry the shaping to the bottom of the piece. The other point to be kept in mind is to trim the paraffin as near to the contained

material as possible without at any time touching it. By having only a slight amount of paraffin around the substance its sections are brought very near together on the slide and thus a more compact mount is secured. If, however, the trimming exposes the material at any point the sectioning is more difficult and of course an injury is done to the specimen.

It is very difficult to orient and trim the block about a specimen which is of about the same color as the paraffin in which it is enclosed. It is therefore helpful, in case of a fixation which does not color the tissue, to tint it in its passage through the higher grades of alcohol with eosin or some other bright color which will serve to mark outlines clearly. If the color is undesirable later it can easily be removed by leaving the sections sufficiently long in alcohol.

**11. Cutting** is the process of removing successive sections of uniform thickness, usually in the form of a ribbon, by the operation of a knife in a machine called a microtome. Not very much that will be helpful in learning proper methods of sectioning can be given by means of verbal description because so much of the facility necessary in this operation comes only through experience. However, there are some general features which may be mentioned and which will be of service. First, the specimen should be so mounted in the holder that the two parallel sides of the paraffin block are strictly parallel with the cutting edge of the knife at the moment of passing it. Next, the holder should be so adjusted that the plane of up and down movement corresponds to the plane through which the section is desired. The knife should be inclined at such an angle to the plane of sectioning that the paraffin strikes it only at the extreme edge. Most knives have a secondary plane of inclination extending only a short distance from the cutting edge and this is the one to be considered in arranging the inclination. On the other hand, the slope of the knife should not be excessive. It should be as nearly vertical as it is possible to get it while avoiding contact anywhere except at the cutting edge. The reason for this adjustment is that the more nearly vertical the knife is the less resistance it offers in passing through the specimen and the more nearly perfect the section it cuts. On the other hand, if the knife is not sufficiently inclined the block drags over the back knife surface below the cutting edge and injures the specimen. Too great an inclination of the knife edge breaks the sections and crumbles them. It is thus obvious that the proper inclination of the knife is an important matter. In order to present the most rigid cutting edge, the knife should be clamped in the holder as near to the specimen as is convenient. The better forms of microtomes have holders which are adjustable in this way. Make all of the adjustments on the microtome to secure the greatest degree of rigidity. Any movement, either of the

knife or of the specimen, aside from that provided purposely, is inimical to good sectioning.

It is hardly necessary to say that only with a perfect cutting edge on a knife can good sections be obtained. Any nicks or irregularities will produce glaring defects in the section.

In cutting sections there appear certain faults which permit of suggestions as to causes, and remedies which may be applied. One of the most common faults is a ribbon which, instead of coming off perfectly straight, with sections of even dimensions, is curved. This form is due to the fact that each section is a little narrower on one edge than the other, which may be due to trimming the block so that the two edges striking the knife are not exactly parallel. Thus each section instead of being a trapezoid, is wedge-shaped. On other occasions the curved form of the ribbon may appear even when the two edges of the block are parallel. This effect may be occasioned by greater density at one side of the block than at the other, in which case the denser half is wider and so produces the same result as converging sides in the block. If the knife edge is not uniformly sharp throughout a difference in section width may thus be produced.

If the sections are much less in diameter than the face of the block from which they are cut, there is a difficulty either with the sharpness of the knife edge or its inclination to the plane of sectioning. In extreme cases the section will be forced into folds that cohere so firmly that it is impossible to straighten the section out completely. If the room temperature is too low, the sections may break across at intervals. This effect is exaggerated if the inclination of the knife is excessive. The nearer the diameter of the section produced is to that of the block from which it is cut, the more perfect is the sectioning. Any excessive disproportion here should lead to an investigation of the causes before the sectioning is continued.

Sometimes the ribbon will cut perfectly for a while and all at once a slit will appear in the sections. In most instances this is caused by the accumulation of material on the edge of the knife which produces a break in each section and so splits the ribbon. Without removing the ribbon from the knife edge this place should be cleaned off with an upward motion of a soft stick. *Always avoid bringing any metal instrument in contact with the knife edge.* If this procedure does not remedy the difficulty, the split is probably caused by a fault in the knife edge, in which event the knife should be slid laterally so as to produce the cut in another place. An air-bubble or other fault in the paraffin block may produce a split ribbon.

One of the most annoying circumstances occurring in paraffin section-

ing is the production of static electricity in the ribbon, which causes it to fly violently towards other objects to which it adheres. This may be avoided by seeing that there is not too great disproportion in density between a specimen and the paraffin, since the electricity is in part produced by differential friction in regions of the block; in other words, the paraffin must be sufficiently hard (58°c.) and the infiltration complete.

Irregularities in the thickness of a section may be caused by lack of rigidity in the knife or in the block, by irregularities in the feed mechanism or by variations in the speed with which the sections are cut. In general it is best to cut at a moderate rate of speed which is kept uniform throughout the process, avoiding any stops if this is possible. Under favorable circumstances the entire ribbon may be cut without breaking and with very little variation in the thickness of the individual sections. Obviously it requires a very fine mechanism to produce several thousand sections of absolutely uniform thickness. Even with the very best machines it is impossible to avoid irregularities unless every condition is favorable. There should not, however, be any marked variation in successive sections. If it should happen that one section is very much thicker than the preceding one it is probably due to an irregularity in the feed of the microtome so that the block adjustment moves forward irregularly, to loose adjustment of knife or specimen, or to too vertical a knife.

These difficulties are a few of the many that may occur in the cutting process and each of them has a definable cause which, when discovered, permits of a remedy. It should, therefore, be the endeavor of the operator to study the difficulty carefully and determine its cause before proceeding with the sectioning operation. When very careful work is done on serial sections it is important to have the ribbons as straight and uniform as it is possible to get them. Much time is lost in passing from one section to another, when cells or structures which should be in alignment are so displaced that they fall without the field of a high power objective. A little time devoted to improvements in the process of sectioning may save a very large amount of work subsequently.

*Manipulation of the Paraffin Ribbon.* As has been noted, the character of the ribbon depends in considerable measure upon the rate and regularity with which the sections are cut. There are also some small manipulations which greatly facilitate the obtaining of a ribbon of the first quality. If it is at all possible the ribbon should be cut without stoppage and wound upon a cylinder entire. This is often difficult of accomplishment and if it becomes necessary to separate the ribbon it should be done, not at the knife edge, but at some little distance from it, leaving a short segment of the ribbon attached. In this way when the cutting is resumed the ribbon will be maintained and the danger of loss from

the first sections rolling up will be avoided. In laying the ribbon down a uniform order should be used habitually. The order followed in writing, e. g., beginning at upper left hand corner of container, etc., is convenient.

In mounting the sections on the glass, care should be taken to see that the polished surface of the ribbon goes next to the surface of the glass. Sections adhere better when mounted in this way. If the block has been trimmed with one inclined lateral margin the ribbon will show a serrate edge which will mark that side of the sections. Thus with the one surface and one edge determined there will be no danger of inverting the order of the sections or of turning the segment upside down. It is of the utmost importance in serial sections to maintain the exactness of seriation.

If a number of blocks are cut at one time it should be the practice to mark upon the container carrying the ribbon the exact serial number which belongs to it. If this is not done it is impossible to avoid confusion. During all the processes it should be the practice to keep attached to the sections their exact designation.

12. **Spreading** is the process of extending sections by heat until all effects of compression in sectioning are removed. If the operation of sectioning has been a success the entire block may appear in a continuous ribbon of sections. In this event cut the series into segments of appropriate length for temporary storage in some convenient container having a clean, smooth paper bottom. For mounting the ribbon is divided into segments of proper length to fall within the limits of a cover glass. In making such divisions it must be remembered that the ribbon expands somewhat in spreading, and allowance should be made for this. If the width of the face of the block is known the number of sections that can conveniently be placed under the cover can be determined by noting how many times the dimension will go into the length of the cover. It is desirable, also, to allow some space at each edge of the cover so that the sections will not lie too near its limits. Having determined the proper length of ribbon segment, take enough of these to occupy the width of the cover glass.

To prepare the glass slip for receiving the sections it is necessary to remove from its surface all foreign material. Commonly this is done by soaking a number of glass slips in battery fluid (p. 77). In place of this some technicians use acid alcohol. Do not touch the glass with any cloth that has not been washed in acid water. Paper towels are clean and convenient for drying. By these means the substances accumulated on the glass surface are removed and it is left chemically clean. *This is an absolute requirement.* As has been indicated previously, the effective-

ness of cleaning may be tested by noting whether the albumen water adheres uniformly over the entire surface. If it should show any tendency to roll up into droplets it is a sure test that the surface is not perfectly clean and the slip should be returned to the battery fluid and left.

Upon the thoroughly clean glass slip is placed a quantity of dilute albumen water (p. 620). On this are arranged the segments of the ribbon, placed so that the first section of the first segment is at the upper left hand corner. The succeeding sections follow each other as do the words in the lines of a printed page. When these segments are properly placed the glass slip is laid upon the table beneath the incandescent lamp which was used to melt the paraffin. At this distance the paraffin ribbon will probably not melt. If it is found that there is a tendency for it to do so the lamp should be lifted somewhat so that there is no danger of overheating the sections. At the same time the temperature should be very near to the melting point for the best results. As soon as the heat begins to affect the ribbons the sections expand and eventually reach a dimension equal to that which they would have in the absence of compression during sectioning. At this time the paraffin, previously showing white, becomes translucent so that against a black background it is hardly visible. *The requirement here is to secure complete extension of the ribbon.* Any irregularities in the ribbon, if not removed at this stage of the process, will remain permanently as ridges or waves in the sections.

When the ribbon is completely extended, the preparation is cooled, the excess albumen water is drawn away, and the ribbons are manipulated by means of pieces of absorbent paper so that they lie perfectly straight and parallel on the slip with the sections of different rows in lines at right angles to their length. When this has been accomplished, absorbent paper is used to draw off the excess fluid and the mounts are placed in covered receptacles so that they may not be exposed to the dust.

It is convenient at this time to mark each of the slides permanently with a distinguishing number, as indicated under "Labeling" (p. 28).

**13. Drying** is a process of removing the remaining water by heat. The slips with the sections upon them are placed in some warm place, such as in the neighborhood of the incandescent lamp, where they are allowed to dry completely. It is best to let them rest in this position for several hours—overnight if possible. At the end of that time the water is completely removed from beneath the sections and they should then lie intimately in contact with the glass surface. Whether they do or not can be determined by turning the slide over and having the light from the window reflected from the surface of the section next to the glass. If it is in immediate contact it will show its complete surface without interruption. Should it, in whole or in part, be separated from the glass by an

air space, this will show as a silvery white area. If there is very much of this separation the section will slip away from the glass after the removal of the paraffin. When spreading has been so incomplete that the sections are nowhere adhering to the glass they may sometimes be refloated on dilute albumen water and further spread, after which they are dried as previously directed. Completion of the spreading process should find the entire series of sections directly absorbed to the glass surface.

In preparation for the succeeding steps a definite arrangement of materials is desirable. Accordingly a brief account of the equipment will be given. Commonly the sections are mounted on glass slips 25 mm.  $\times$  75 mm., but embryological mounts are often made upon slips as large as 50 mm.  $\times$  75 mm. The size of the vessels employed is accordingly to be determined by the dimensions of the slips. There are two general means utilized for carrying the preparations through the various solutions. One of these is to place them in a proper sized glass box with divisions for holding the slides upright and allow them to remain in this vessel, to which the different solutions are successively added and withdrawn. The other is to lift the slides from one vessel to another containing appropriate media or reagents. If large numbers of slides of a similar character are carried through at one time the former method is better because it avoids handling them individually.

If, on the contrary, a small number of slides or those of diversified character are to be handled, they must be treated individually and under these circumstances the more convenient way is to transfer them from one vessel to another. For this purpose a jar of distilled water and a series of alcohols, each contained in its separate tall Stender dish, are arranged in order from 30 per cent to 95 per cent, followed by a Stender dish containing a mixture of equal parts of 95 per cent alcohol and xylol and finally a jar of pure xylol. Since the slides are run both up and down the series, there is some advantage in having two jars of xylol, one used in the ascending series and the other in the descending. If the sections are to be stained on the slide there should be Stender dishes with appropriate solutions.

**14. Decerating** is the process of removing paraffin from the sections. Until the sections are quite firmly adherent to the glass the paraffin is necessary to support the tissue but when thus attached they no longer require the paraffin, and, since it interferes with the further operations of staining and mounting, it must be removed. This is done most conveniently by plunging the entire slide into a jar of xylol, which almost at once dissolves the paraffin. If large numbers of similar slides are carried through in the glass boxes, the only difference will be that the fluids are



successively applied to the slides in the one container. *After the removal of the paraffin the sections must never be allowed to dry.*

**15. Preparation for Staining.** There are no stains commonly used which are dissolved in the medium employed to remove the paraffin, so the xylol must be displaced before staining is possible. The first step then is to dissolve out the xylol with 95 per cent alcohol from which the sections are directly transferred to the stain, if it is an alcoholic one. In case the stain is aqueous, as it commonly is, it is necessary to hydrate the sections by running them down through graded alcohols from 95 per cent.

Transfer the slides successively into the jars containing a mixture of alcohol and xylol and down through the descending series of alcohols to water. The slides should remain in each bath of alcohol only long enough to secure the substitution of that strength for the one preceding. This is indicated by the uniform appearance of the fluid as the slide is lifted from the jar. So long as there is a difference between the strength of the alcohol in the sections and that of the solution, diffusion currents will appear. In proceeding down the series it is not necessary to be particularly careful about drawing off the alcohol of the higher concentration since it only serves to increase the percentage already present. However, in proceeding in the reverse direction, in order to avoid diluting the alcohols of higher concentration it is best to remove the excess fluid with absorbent paper. A paper towel to which the slides are touched is convenient for this purpose.

**16. Staining.** This is the process of increasing the visibility and contrast of cell and tissue parts by their differential reactions to dyes (for details relating to staining see p. 573). According to the method of their application stains fall into two classes, first, the progressive stains which are operated by leaving the sections exposed to their action until the desired degree of coloration is reached; and, second, regressive stains which are allowed to act until overstaining is accomplished, after which the desired degree of differentiation is brought about by removal of the excess coloration. It will serve the purposes of the present outline to indicate how representative examples of these two methods may be employed.

Common stains are primarily nuclear stains and of these hematoxylin is probably the best. It may be applied in either of the two ways indicated. As an example of direct staining we may consider the application of Delafield's (p. 613) hematoxylin or Mayer's (p. 469) haem-alum.

The sections with the paraffin removed and hydrated are ready to be placed immediately in the staining agent. This is preferably contained in a convenient-shaped glass vessel so that the slides remain in a vertical position. Into this is placed a dilute solution of Delafield's hematoxylin.

As a general principle it should be recognized that the more dilute the solution the longer the operation and more precise the result of staining. If rapid effect is desired the stain should be used in a more concentrated form. The only way that the effect of the stain can be determined is by examination of the sections under the microscope. Only in exceptional instances, for specifically indicated materials, is it possible to give the exact time for the staining operation. It is suggested that as a trial period the sections of liver be stained with one-fourth strength Delafield, 5 to 10 minutes. When by examination it is found that the desired structures are properly colored, the operation of the stain may be terminated by putting the slides in tap water for at least ten minutes, where the excess of stain is removed. In the event of over staining, this excess may be reduced by placing the sections for a short time in acid alcohol. The color of the stain depends upon its acidity, being red if acid, or blue if alkaline. Since tap water is slightly alkaline, washing in this medium gives an agreeable blue. Washing should always be thorough after staining and differentiating in order to secure permanence and precision of the stain. Mayer's haem-alum, although not so generally used as Delafield's mixture, produces a more exact and delicate result, even when used undiluted.

As an example of the regressive stain, the iron hematoxylin method of Heidenhain may be instanced. In this process the sections are first mordanted for a period of from two to twenty-four hours in a 4 per cent solution of ferric alum. They are rinsed for a sufficient length of time to remove the excess iron alum solution and are then placed in a  $\frac{1}{2}$  per cent aqueous solution of hematoxylin where they remain for a period of time approximately equivalent to that employed in mordanting them. At the end of this operation they are completely black, showing practically no differentiation whatever. To accomplish the desired degree of destaining they are rinsed quickly (thirty seconds) and returned to the mordanting solution which now acts as a differentiator. Gradually the hematoxylin is dissolved out, beginning with the cytosome and proceeding to the nucleus. The last substance to lose the stain is the chromatin. It is possible by this method to secure the most extreme degree of selectivity between different portions even of the chromatin, and it is one of the most valuable of fine cytological methods. The quality of the stain is improved by frequently rinsing the slides in water as they are examined under the microscope during the process of differentiation. The longer operation of staining gives a greater range of coloration which can be thus extended from the nucleus to the cytosome, bringing out such structures as centrosomes which are lost in the more rapid

process. In some cases satisfactory results can be secured by mordanting and staining for periods within an hour.

After differentiation is complete the excess of iron alum must be very carefully removed by washing an hour in running water. The resultant color of the structure depends in some measure upon the age and quality of the hematoxylin and ferric alum solutions. Fresh solutions give a bright blue effect, while those that have been repeatedly used range through black to a rusty green color. The character of the wash water also has an effect upon the color. At the end of either progressive or regressive methods with hematoxylin we have a result showing the nucleus more densely colored than any other portion of the cell, while within the nucleus the chromatin is strongly distinguished by its greater affinity for this coloring agent.

**17. Counterstaining** is a process of adding one or more additional stains, which, by contrast of color and selectivity to the primary stain, emphasize distinctions between cell and tissue elements. In some cases it is desirable to thus differentiate the parts of the tissues or cells by the selective influence of different kinds or colors of stains. If hematoxylin has been used as a nuclear stain it is a common practice to emphasize the cytoplasmic structures by means of such counterstains as eosin or Congo red, which both in color and selectivity, contrast with hematoxylin. The easiest way of applying such counterstains is to have an alcoholic solution of them and pass the sections through it in the process of dehydration. Care should be exercised not to vitiate the bright blue color of the hematoxylin by admixture of the eosin, which is so general a stain that it will take in any part of the cell. In most instances the use of a counterstain is of doubtful value, since there are rarely revealed structures which are not sufficiently differentiated by the primary nuclear stain.

A counterstain may be applied to the piece of liver as indicated.

**18. Dehydration.** Most staining agents are aqueous, and since the mounting medium is commonly a balsam, the water must be removed by the usual method of dehydration, running the sections up through grades of alcohol into 95 per cent and even absolute. While it is perhaps not necessary always to take this precaution, in exceptional instances the rapid transfer of sections from water to 95 per cent alcohol may injuriously affect them. A slight expenditure of time in running up through grades of alcohol will obviate this and as a matter of routine it seems to be worth while to employ the gradual method of dehydrating except where certain stains are too readily removed by the alcohols.

**19. Clearing.** The sections, after having been dehydrated, are prepared for enclosure in balsam by passing them through some clearing medium, commonly xylol. It is necessary to obtain a complete removal

both of alcohol and of the water which preceded it; otherwise the sections will become clouded when placed in the balsamic mounting medium. Clearing with one of the essential oils in order to avoid the condensation of water by the evaporation of the xylol is helpful. A rapid but thorough rinsing out of the clearing oil by xylol should occur.

**20. Mounting** is a process of enclosing the prepared sections in some medium which will preserve them indefinitely under suitable conditions for microscopical observation. The slide is removed from the clearing medium, the excess of this being wiped from the surface with absorbent paper. Upon the center of the group of sections is placed a sufficient amount of gum damar which has been dissolved in xylol, to occupy the space between the cover and the slip. Only experience will indicate how much of this should be used, but endeavor not to add an excess of balsam because it makes a difficulty later in cleaning the slide. On the other hand, see that there is no space left beneath the cover not filled with the mounting medium. A perfectly clean cover glass is held by forceps and gently lowered obliquely upon the surface of the mounting medium. If the contact is made too rapidly, small air bubbles are almost certain to be enclosed. By careful manipulation, however, the cover can be placed upon the balsam so as to completely exclude all air. The cover is then lightly pressed down so that it is brought into contact with the sections beneath. It is quite undesirable, especially for high power observations, to have any excess quantity of balsam between the section and the cover glass. Any considerable surplus of balsam should be carefully wiped off with absorbent paper after which the slides are ready for the next process, that of drying.

**21. Drying** is the process of removing the solvent from the resinous mounting medium so that the sections are left enclosed in a solid substance. In order to do this the slides are placed in a horizontal position in some warm place where evaporation is facilitated. They should remain there until the balsam has become thoroughly hard, so that pressure upon the cover glass will not cause it to move over the sections. Any excess of heat, however, should be avoided, since it may injure the tissue, while the too-rapid evaporation of the solvent will cause open spaces beneath the cover glass and may even injuriously affect the quality and color of the mounting medium.

After the damar is thoroughly hardened, the excess is removed from around the edges of the cover by scraping. One should be careful to avoid contact between the knife which is used for this purpose and the edge of the cover, which may thereby be broken. Finally, the remaining balsam outside the edges of the cover is removed by dipping the slide in xylol and wiping it carefully with absorbent paper or cloth.

**22. Labeling.** If any number of slides have been carried through together each of them should bear a distinguishing number. Preferably this is the number which marks the lot, supplemented by the number of the slide in the series. The best way to indicate permanently the source of the material is to scratch its number upon the surface of the glass slip with a diamond or engraver's carborundum pencil point. In large collections which are completely catalogued this is a sufficient method of labeling individual slides, but in cases where these are consulted by many people it is helpful to have in addition a label which indicates the nature of the material and the character of the processes through which it has passed. Commonly this is done by writing or printing the data desired upon paper labels of proper size and then attaching these by means of gum to the ends of the slides. It is best to have a regular system of labeling, and most workers place the main label at the left end of the slide. Upon this there may be a complete record of the source and character of the material, the nature of the processes through which it has passed, the date, and the name of the preparator. A complete preparation of this sort is properly designated as a microscopical slide. Unfortunately this term has become confused with the term "slip" which should be applied to the piece of glass upon which the object is mounted.

**23. Storing.** There are various methods for permanent storage of slides, with characteristic advantages and disadvantages. The most common method is to place the slides in wooden boxes bearing racks into which the ends of the slides fit. These are of various sizes, but commonly one holding 25 slides is used. There are other boxes, usually made from cardboard, in which the slides are laid horizontally protected by a cover which falls over them.

One of the very best methods is a recent contrivance. This is an envelope without a flap, made of heavy paper, such as "Paperoid."<sup>2</sup> These envelopes are  $3 \times 5$  inches in size, sealed at the ends, and divided into four compartments by uniting the front and back with staples. The upper portion of the front is cut off  $\frac{1}{2}$  inch shorter than the back so that when the slides are inserted into the compartments the label is exposed. If labeling has been done with a diamond, this provides sufficient space so that the designating numbers are visible when the group of four slides is examined. These containers, holding four slides each, may be inserted vertically into any of the devices used for filing  $3 \times 5$  library cards and may there be combined with records and drawings, pertaining to them individually, which have been placed upon library cards of the same dimensions. This system has many advantages, since it occupies only a limited space, leaves no vacancies, protects the slides thoroughly so that

<sup>2</sup>Hance, R. T. *Anat. Rec.*, 28: No. 5, 1924.

they may be shipped without any danger of breakage, and in the event that the container is dropped on the floor, protects them from injury. It has the very great advantage of extreme flexibility, providing a means not only for storing a complete collection readily, but for withdrawing all of the slides relating to a particular species or subject without necessity for sorting. Altogether it is the best arrangement that has been so far devised for storing and consulting collections of slides.

If slides are used by more than one person their loss may be prevented and the convenience of consultants served by inserting into the space from which each slide is removed a card of the same size upon which is recorded its number, the date of withdrawal and the name of the person having it. To make such an entry requires little time and trouble and will often save valuable specimens.

## II. Collodion Method

1-6. In the application of the collodion<sup>3</sup> method the steps follow in order as outlined in the paraffin method from 1-6 inclusive. Dehydration must be complete since the collodion is dissolved in the mixture of absolute alcohol and ether in equal parts. Treatment in absolute alcohol is therefore required.

7. Since the material goes from absolute or from the alcohol-ether mixture directly into the infiltrating medium, item 7 of the paraffin method "Clearing" is omitted.

8. **Infiltration.** This is accomplished without the application of heat by placing the thoroughly dehydrated material directly into a thin syrupy solution of trinitrocellulose. The time required for infiltration depends upon the size and character of the pieces of tissue. The object to be attained is to displace the absolute alcohol by thin collodion. When this has been done the next thing is to secure a gradual evaporation of the solvent so that the tissue becomes infiltrated with a thicker and thicker concentration of the collodion. This stage may require many days or even weeks if the specimen is large. It is necessary to provide so gradual an evaporation that concentration is uniform throughout the mass. When the infiltrating medium becomes concentrated almost to a gel the material is ready for the next process.

9. **Imbedding and Hardening.** The piece of tissue is brought into a paper box or vessel of appropriate size filled with thick collodion. This is then put aside under a small bell jar and evaporation allowed to con-

<sup>3</sup> Collodion is a solution of a trinitrocellulose made from cotton. Other forms of the compound recently developed have the special names, celloidin, parlodion, etc. Collodion is here used in a generic sense.

tinue somewhat more rapidly until the syrupy fluid has become so hardened that it will retain an impression of the finger nail. Care must be exercised that evaporation is not so rapid that the concentration of the collodion outside of the tissue is greatly different from that within. A final hardening of the collodion is accomplished either by placing the block in 80 per cent alcohol or by exposing it to the vapors of chloroform. Sometimes hardening is secured by treatment with chloroform followed by 80 per cent alcohol. As a result of the treatment the collodion is now of such a consistency that it will readily section. If the piece of tissue is small enough infiltration and imbedding may conveniently be carried out in an appropriate sized shell vial from which the cork is occasionally removed for brief intervals to regulate the rate of evaporation. When the medium is sufficiently stiff the object is oriented to be cut parallel with the bottom of the vial and a little later the mass is loosened from the vial. When stiff enough finally it may be removed from the container.

**10. Blocking and Trimming.** The mass of hardened collodion with the contained tissue is mounted upon a proper support with thick collodion which is set after a few minutes drying by immersion in 80 per cent alcohol for three or four hours. Fiberoid blocks are convenient for this purpose. Small floor tiles are also used. It is not necessary, as in the case of paraffin, to trim the block to any particular shape so that the only requirement is to remove any excess of the mass about the tissue.

**11. Cutting.** Instead of cutting the sections with a dry knife placed at right angles to the direction of movement, the collodion sections are cut with a wet knife adjusted at a narrow angle to the direction of movement. Cutting is facilitated by having a conveniently placed vessel which drops 70 per cent alcohol upon the knife's surface at such a rate that there is always a small pool of fluid at the point where the section comes off. The manner of sectioning varies with the microtome used, but should be accomplished with a firm uniform movement. The sections are most conveniently handled by a moistened camel's hair brush. If they are to be mounted serially they are moved into position successively as they are cut until a sufficient number has been accumulated in proper arrangement for mounting. They are removed to the clean glass slip by placing a thin piece of tissue paper over them and pressing this down until the sections adhere. By carefully sliding the piece of paper and the adhering sections off the knife they can then be transferred to the glass slip. Press the sections into contact with the glass surface by rolling them with some cylindrical object, after having interposed several layers of absorbent paper, when they will adhere sufficiently so that the paper may then be removed. The sections may be caused to adhere permanently to the glass by pouring over them the vapor of ether which softens the col-

iodion, or by action of clove oil which has a slightly solvent effect. Remove the oil after five minutes with 95 per cent alcohol. Mayer's albumen applied in a very thin layer to the glass will also cause the sections to adhere. After the sections are arranged, coagulate the albumen with 95 per cent alcohol.

12-15. These steps of the paraffin method are omitted since the sections are spread by the above-described operation. Drying is omitted because the sections already adhere to the glass and because it is not possible to dry the collodion sections without injuring them. The imbedding medium is not removed at any time in the collodion method.

16. **Staining.** Staining may be done after the sections are attached to the glass as in the paraffin method, but more commonly for histological work the material is either stained in toto or the individual sections are floated in the stain and thus prepared before attaching them to the glass. This is not feasible, however, in case of serial sections. The staining of the attached sections is carried on according to the manner described in the paraffin method.

17-23. These steps are performed as described in the paraffin method.

#### *Modifications of the Collodion Method*

*Gilson's Rapid Process.* It is sometimes necessary to secure sections by the collodion method more rapidly than is possible with the usual procedure. The objects are infiltrated with collodion while heat is applied by dipping the tube containing the material into melted paraffin. Here the collodion boils and produces a very rapid evaporation of the solvent. After it has reached a consistency which will just permit it to be poured from the vessel the specimen is removed and placed upon a block of hardened collodion and is then surrounded by the thickened medium. This is then hardened in chloroform and cleared in cedar oil. After the object has been mounted on the microtome the sections are cut with a knife moistened with cedar oil. If the material has been stained in toto the sections are then ready to mount.

*The Dry Cutting Method.* This is somewhat similar to the method of Gilson but with slow infiltration. After the object has been infiltrated it is hardened as usual with chloroform vapor and is then placed in the mixture suggested by Gilson; that is, equal parts of chloroform and cedar oil. To this, cedar oil is added by degrees until the mixture is replaced by pure cedar oil. The block is then ready for cutting after being properly mounted. It is cut dry, requiring only that the knife be moistened with the cedar oil in order to prevent the sections from tearing. Sections are ready for mounting if they have previously been stained. In case it is desired to stain the material it will, of course, be necessary to run the sections down through alcohols until the proper concentration to meet that of the stain is reached.

The dry method has several advantages over the ordinary one. The sections



being infiltrated with the oil are not so difficult to handle and the blocks from which they are cut may be preserved indefinitely because of the presence of the oil.

### III. Freezing Method

This is a method not wholly adapted to precise results, but at the same time is one of very great convenience. It is rapid and easy of application and gives results sufficiently good for diagnostic purposes. It is, in fact, most often used by diagnosticians who are obliged to get information with regard to the character of a tissue as rapidly as possible. It is also useful in studies of cell constituents when ordinary methods would destroy or injure the substance to be studied.

Perfectly fresh tissue, just removed from the animal, may be placed upon the freezing microtome and sections cut from it as soon as it has been frozen. More frequently, however, the material is fixed and is then placed in a medium which is better adapted to sectioning than plain water ice. Of the fixatives most commonly used 4 per cent or 5 per cent formol is best. If there is need for haste, after fixation the material may be frozen in the fixative but commonly it is better to wash this out and substitute for it a medium made by combining a saturated solution of sugar, 3 parts added to 5 parts of mucilage prepared by dissolving 60 gm. of gum acacia in 80 c.c. distilled water. The tissue is infiltrated with this medium and then removed to the holder on the microtome where it is covered by a quantity of the mucilage. It is frozen in this fluid and sections are then cut.

Frozen sections may be cut with an ordinary microtome, but more frequently a special piece of apparatus attaching directly to a carbon dioxide cylinder is used. In this case a knife in the form of a chisel is employed to cut the sections. As in the case of other section methods the skill required can be gained only by practice but there are some suggestions which may be of service. In order to prevent the knife from melting the mass as it passes over it, it should be kept cold. This also prevents the sections from sticking to the knife and becoming torn. Speed is an essential requirement in sectioning with this method. Sections are handled with a camel's hair brush like the collodion sections.

After the sections are cut the gum solution is removed by immersion in water and then they are ready for staining. The common method is with Delafield's hematoxylin which may be followed by eosin for a counterstain. Following this the sections are treated as in the other methods, mounting them finally in damar.

#### IV. Smear Method

The essence of the smear method is to form a very thin film of the tissue upon a glass surface so that the elements are individually distinguishable. This is accomplished in three ways. The first is by placing between two clean cover glasses a small quantity of the material to be treated. The cover glasses are then pressed together so as to dispose the material uniformly between them. If the substance is a fluid like blood no pressure is required, but in most other instances a certain amount is necessary. This is a delicate point in manipulation and the extent of pressure can be determined only by experiment. The end to be aimed at in most cases is to manipulate the materials so as to separate the cells and to avoid crushing them. In some instances, however, it is desirable even to rupture the cell in order to distribute its formed elements, like the chromosomes, so that they may be more readily observed. Except in the case of fluids, it is impossible to secure absolute uniformity in the thickness of the film and in some respects this is an advantage because various degrees of extension of cell contents may be found in different regions of the preparation.

Assuming that the proper distribution of the elements has been obtained, the next step is to slide the two covers apart rapidly and uniformly. Two precautions should be observed; First, to separate the covers exactly in the plane of their contact,—they should be slid apart and not pulled apart. Second, the movement of separation should be uniform. If it is not there will be areas of different density according to the speed with which the separation has proceeded. Even with the best of manipulation, however, it is rarely the case that the films on the two covers are equally good. If it should happen that the tissue is of such character that there are residual masses of considerable thickness it will be desirable to remove these after fixation.

The subsequent treatment of the film depends upon the character of the material. In the case of blood the films are fixed by drying and there are other instances where this is possible. Some materials, however, are entirely ruined by allowing any evaporation to take place and in these cases the films should be dropped immediately upon a fixing fluid after separation of the cover glasses. What will happen to any given kind of cell can be determined by experience alone. As an example of the variation of behavior in this respect it may be stated that the cells of Hemipteran testes are quite uninjured by drying while those of the Orthoptera are completely ruined.

Another method of obtaining a film is exemplified in the ordinary

process of making blood smears. Here a drop of blood is picked up on the end of a clean glass microscopical slip and this is at once applied to the surface of another slip. By a uniform and fairly rapid movement the blood is distributed over the surface of the second slip by moving the first along its length. There are several precautions which should be observed in this procedure. The edge of the slip chosen to carry the drop of blood should be perfectly straight so that it will lie in contact evenly with the surface of the other upon which the blood film is to be formed. Also it is most desirable that the second slip have as uniform a face as possible. If there are variations in these two matters the film will be of unequal thickness. Likewise it is necessary in spreading the film to proceed with a uniform movement; otherwise inequalities in thickness will be produced. The best method of operating is to lay the second slip upon the surface of the table, applying to it, near one end the one with the blood, at an angle of about  $45^{\circ}$ . Hold the glass slip carrying the blood between the thumb and first finger, and support the hand on the table by the tips of the remaining fingers. In this position the movement of the carrier can be made uniform in pressure and rate. (For a detailed account of the use of this method see the section on Blood, p. 325.)

A variant of this second method of smearing may be employed in the case of such tissues as testicular material where it is desired to get films of the germ cells. Where this is possible a film is produced by taking a piece of the tissue with a pair of forceps and wiping it over the surface of the glass slip. In this way a layer of the contained germ cells is left behind. This is a method which has been used very successfully in the case of Hemipteran germ cells, gland cells, bone marrow, etc. A third method used with plant cells is described on p. 165, Chapter III. Films obtained in any of these ways, after fixation and proper washing, may be stained in the same way that sections are and mounted properly with damar.

The advantages of the smear method are that, with it, preparations may be rapidly secured in which the entire elements are preserved and often in such a form that details of structure are unusually well displayed. The disadvantages lie in the fact that normal relations of some elements are disturbed and, by reason of variations of pressure and drying, cell elements are differentially affected. Only when necessary should observations based upon smears be used unsupported by a study of the same material in sections.

*A special refinement of the cover glass method, adapted especially to secure perfect films for blood studies is thus described by Isaacs.*

Cover glasses which have been soaked in nitric acid over night or in warm nitric acid for about one-half hour, are thoroughly washed in running water. A funnel held upright and attached by means of a rubber tube to a faucet serves

as a convenient washing device. The washed cover glasses are placed in 95 per cent alcohol and kept there until they are to be used. With a pair of forceps they are transferred from the alcohol to a wide mouthed bottle containing either ether or acetone. From the ether or acetone they are transferred to a piece of filter paper and allowed to dry. With the forceps they can be placed on a piece of smooth writing paper lying on a flat table. To polish the surface, the finger is placed on the glass, and with firm pressure the cover slip may be moved back and forth over the paper. After the cover glasses have been polished, they should be brushed with a large grease-free camel's hair brush to remove any lint or dust. In this way the side touching the finger becomes covered with grease, but the side nearest the paper will remain grease-free for several minutes. The blood is drawn in the same way as for making blood counts and when a drop about 3 mm. in diameter has accumulated, one of the cover glasses is touched to it and a drop about 2 mm. in diameter is picked up. The cover glass is then placed in contact with another clean and polished cover glass and the drop allowed to spread until it has almost reached the edges of the glasses. While it is still very slowly spreading, the two cover glasses are drawn apart with a short, sharp snap. If the cover glasses are placed over each other so that the corners project, a single corner of each cover glass may be used as a place for holding them.

The blood films are allowed to dry at room temperature for at least three minutes and longer if time is available. If the staining process is begun before the blood is dry, the blood will sometimes come off the cover glasses. The best results are obtained if the films are stained within twenty-four hours after being made.

## V. Stretching Method

Certain membranes in the body, like the omentum, are best prepared by this method. A convenient way of securing such preparations is to use two rubber rings which fit one within the other. By stretching the membrane over the smaller ring and then passing the large one over the smaller, the tissue is tightly stretched. The membrane is then trimmed around with scissors and in this condition can be handled very much like a section. The subsequent treatment of the preparation depends upon the object in view. If it is to be stained and mounted in the ordinary manner the series of steps indicated in the paraffin method would be followed with the exception of steps 8 to 15. Other material, like subcutaneous tissue, may be prepared by a modification of the method.

*Subcutaneous Tissue Spreads.* Loose subcutaneous tissue is found in any vertebrate just under the skin, between that and the muscles. It is usually taken from the abdominal or inguinal regions.

After the selected area is shaved, an incision is made in the skin and a very small piece of subcutaneous tissue is snipped off with scissors. This piece is

placed on a cover glass and quickly spread out as flat and as thin as possible by means of two teasing needles, taking care, however, not to tear it to the extent of injuring the cells. It is an advantage for two people to work together in doing this, using two needles each. The cover glass is then floated on fixing fluid, the tissue side being down. All the above manipulations must be done quickly before the tissue dries, and it facilitates handling the cover glass if the teasing is done with the cover glass over a black background and supported by a Petri dish which is bottom side up. (Slider.)

There are certain other more rigid structures which may be mounted entire like these more delicate membranes. An object of very great value for microscopical observation, for instance, is the gill plate of certain amphibia, like *Amblystoma*. These structures have sufficient rigidity so that they may be handled like collodion sections and treated very much in the same manner. They do not require the stretching process but are treated, otherwise, essentially the same.

The advantage of a method of this character is that the structures are all preserved in their normal relations while at the same time one has almost the same conditions of thickness as is found in sections. In cases where cells are not cut through, however, the staining is in some ways less precise. However, when a regressive method like Heidenhain's iron hematoxylin is used, this is not a serious difficulty. In very thin membranes like the amnion, cytological details are well presented.

## VI. Teasing Method

Certain fibrous tissues like white fibrous, yellow elastic, and muscle, are advantageously prepared according to this method. Before teasing it is desirable to fix in such a way as to get a differential action upon the interstitial substances. For this purpose dilute fixatives are commonly utilized so that a partial maceration precedes the actual act of teasing. After the material has been properly fixed, it is taken in small pieces, either on the glass slip where it is later to be mounted, or in a watch glass, and combed out by the use of two dissecting needles. Care should be exercised not to break the fibers across, but rather to comb along their length so that the cement substance is removed, leaving the fibers free at their ends.

By a sufficient degree of maceration the real act of teasing is much reduced in extent, and it is often possible to almost entirely dissociate the elements previous to their mechanical separation. It is sometimes advantageous to stain the material before teasing, but if this is not done a stain may be dropped upon the material as it rests on the slip or in the watch glass, and its action watched under a microscope. The further operations

of differentiating, dehydrating, and clearing are also accomplished preferably on the slip where the material is to be mounted.

Difficulties in teasing that may be encountered are largely due to the lack of proper treatment in fixing, and it is often better to take additional time in the earlier steps of the process rather than to try to separate the tightly joined fibers by force with the needles. In general, what will be secured by an operation of this sort is a number of masses of material from which free elements project more or less. The limitations of this method are obvious and while it is useful in certain cases, it is inferior to many of the others as a general method.

### VII. Grinding Method

This method has very special applications which will be considered in detail under the topics of bone and teeth. It is a physical method which involves the use of abrasives upon hard structures. Appropriate-sized pieces of tissue are fastened by cement to the glass upon which they are to be mounted, and then by grinding and polishing, the thickness is reduced sufficiently so that the light will pass through. After washing, cleaning and drying the surface the specimen is mounted in balsam beneath a cover (p. 353).

### VIII. Macerating

All the preceding non-section methods of making microscopical preparations are physical in character, but maceration depends upon chemical action. As has been explained, it is made possible by the differential effect of weak fixatives upon interstitial or cement substances. An appropriate piece of tissue is placed in the weak fixative and allowed to remain sufficiently long for the reagent to dissolve the interstitial substance and then, upon shaking in a bottle, the elements may be freed from each other and made ready for further treatment. This will depend upon the fixative employed, but assuming that any ordinary one has been used, the next step would of course be the removal of the excess fixative by washing. The various steps of the paraffin method can then be followed, leaving out those from 8 to 15.

The several processes can best be performed in a small shell vial, because the elements of the tissue, either by gravitation or by centrifuging, may be brought to the bottom of the tube after each treatment, and the supernatant fluid can be withdrawn and that required for the next step added. It is even possible, and in some cases very desirable, to carry the

cells clear through into the balsam where they may be kept indefinitely and removed with sufficient medium to the slip for mounting.

The advantage of the maceration process is that entire elements are obtained. The disadvantage is that because of the use of a weak fixative the elements of the cells are not nearly so accurately preserved as they would be with a normal fixation. In most cases, therefore, if it is desired to obtain detailed information with regard to the structure of intracellular elements, the maceration process should not be employed.

### IX. Recent Modifications of the Paraffin Method

The whole process of making paraffin sections can be much shortened and the results even improved by the use of either butyl alcohol or of dioxan. These fluids are miscible with water or low grades of alcohol on the one hand and dissolve in paraffin on the other, and thus eliminate one step, that of clearing, which also is one of the most harmful to many tissues.

**Normal or Iso-butyl Alcohol.** Material fixed, washed and run up to 50 per cent ethyl alcohol is brought into butyl alcohol. If the tissues are delicate the transfer to butyl alcohol can be made through mixtures of ascending strength until absolute butyl is reached. The length of time in each mixture will depend upon the size and character of the tissue, but an hour should be sufficient in ordinary cases. Final dehydration in absolute butyl may require twenty-four hours. It may be an advantage to use mixtures of paraffin in butyl alcohol, beginning at room temperature and finishing with larger proportions of paraffin at higher temperatures. Finally the tissue is transferred to melted paraffin for complete infiltration. This will require a longer term than after a clearing in xylol, because of the lesser solubility of paraffin in butyl alcohol.

**Dioxan.** Dioxan, dimethyl dioxide,  $O(CH_2CH_2)_2O$ , is a clear colorless fluid with a slight and characteristic ethereal odor. Its specific gravity is 1.040, and it volatilizes at a temperature of  $101^\circ C$ . It is miscible with water, alcohols, xylol and oils, and dissolves paraffin (slightly), resins, gums, hematoxylin, mercuric chloride, and picric acid. So far as tried it does not dissolve aniline dyes, except Sudan reds or other fat-soluble dyes, except in the presence of water.

Dioxan is an almost ideal reagent because of its long range of solubilities and because it has no injurious effect on tissues. It is possible to carry material through all the stages up to sectioning without leaving dioxan. In this case a fixative containing dioxan is used, washing is done in the same medium and the tissue is finally transferred to melted paraffin. A good fixative containing dioxan is the following:

Sat. aq. sol. of picric acid .....	5 parts
Acetic acid, glacial .....	1 part
Dioxan .....	4 parts

Another not involving the use of any water, is:

Sat. sol. of picric acid in dioxan .....	4 parts
Acetic acid .....	1 part
Absolute alcohol .....	4 parts

Also:

Dioxan .....	6 parts
Chloroform .....	2 parts
Acetic acid, glacial .....	1 part
Sat. sol. picric acid in dioxan .....	80 per cent
Paraldehyde .....	10 per cent
Acetic acid .....	5 per cent

Graupner and Weissberger<sup>4</sup> recommend the following fluid:

Dioxan .....	80 per cent
Methyl alcohol .....	20 per cent
Paraldehyde .....	2 per cent
Acetic acid .....	5 per cent

The proportions of these ingredients may be changed to meet the conditions in various tissues.<sup>5</sup>

Dioxan seems to have no harmful effects upon tissues which remain in it for long periods of time. So far as experience has gone, they may lie in it indefinitely without injury. They also seem less likely to be altered during infiltration than after clearing with xylol, although the period required in the melted paraffin is longer.

Altogether this reagent promises to be one of the most useful so far discovered. Not only may it be used during fixation, dehydration and clearing, but it may serve as a solvent for the excellent mounting medium, sandrac, so that the tissue need never leave dioxan anywhere in the process except during infiltration and staining. A schedule for a piece of liver, used as an example previously, would be

1. Fix in dioxan, picric acid, acetic, alcohol mixture 2-24 hrs.
2. Wash in dioxan, 72 hrs.
3. Infiltrate in paraffin, 6 hrs.
4. Mount sections in sandrac, camsal, dioxan mixture.

If tissues have been fixed in aqueous fixatives and carried up in the

<sup>4</sup> *Zool. Anz.*, 102: 39-44, 1933.

<sup>5</sup> These fluids are all very vigorous and do not work well on delicate material.



usual way to 70 per cent alcohol they may be dehydrated finally in dioxan and then taken into paraffin. Material fixed in Carnoy and other alcoholic fixatives can go directly into dioxan. It is essential to have the final bath free of water before the tissue goes into paraffin. To make sure of this a small quantity of calcium oxide, calcium chloride or desiccated copper sulphate may be kept at the bottom of the container in which the specimen is floated.

### X. A New Mounting Medium

The mounting medium referred to is prepared by dissolving selected, clean tears of sandrac in dioxan, filtering, concentrating, and adding 1 part of camsal to 9 of the sandrac solution. Camsal is made by mixing camphor and salol in such proportions that a little camphor remains undissolved in the resulting fluid. The mixture of sandrac solution and camsal is filtered, if necessary, and further concentrated until of a proper density for mounting specimens. This is essentially the same medium as euparal and specimens may be carried into it either from alcohol or dioxan without clearing. It dries quickly, does not chip or crack, is clear and transparent and promises to be a most useful medium. The refractive index is somewhat lower than that of damar or Canada balsam which, for delicate cytological details, is an advantage.

### XI. Sectioning Refractory Tissues in Paraffin

The question of sectioning yolk-laden eggs, woody tissues, and other refractory objects in paraffin has always been a difficult one. The discovery that soaking the imbedded object in water restores its plasticity has largely solved the problem and has made possible the cutting of serial sections of such difficult objects as insect eggs. The practical procedure is to trim the paraffin block until the imbedded tissue is exposed on one side and then to soak it in water for several hours. When the material is hydrated in this way it loses the friability and brittleness which it gained during dehydration and clearing, and it then cuts much like fresh tissue. The dioxan technique does not always require hydration of the tissue.

PART II

SPECIAL METHODS



## CHAPTER II

### METHODS FOR THE STUDY OF FRESH MATERIAL

MICRODISSECTION 43. MICROINJECTION 51. TECHNIQUE FOR CELLULAR PHENOMENA 62. VITAL STAINS 110. SUPRAVITAL STAINS 117.

#### MICRODISSECTION

##### 1. Free-hand Manipulations

SVEN HÖRSTADIUS

This section deals with methods for operating free-hand under comparatively low magnifications. The operations are preferably performed under a dissecting microscope because of the erect image it gives. The magnification of such a microscope does not exceed 60-100.

Only a short account, drawn from the author's personal experiences with methods generally in use, will be given. A more detailed description of some of the methods will be found in Spemann (1923)<sup>1</sup> and Mangold (1928).<sup>2</sup> However, great detail is not required in all cases, since many modifications must be made according to the problem for which a given method is used.

Fine knives or needles, specially sharpened on good hones, have proved to be serviceable even for such minute operations as the separation of blastomeres (Zoja, 1895)<sup>3</sup> or the division of the uncleaved egg of the sea-urchin (Delage, 1899).<sup>4</sup> A decided advance, however, was made when Spemann (1906)<sup>5</sup> suggested the use of fine glass needles or hairs for operations both on the Amphibian and the Echinoderm egg.

A glass tube or rod of 5-6 mm. in diameter and 15-18 cm. in length is seized at both ends, its middle part well heated in a gas flame and, outside the flame, quickly drawn out as far as the arms reach. When broken to a suitable length the larger ends are to be used as handles (Figs. 2-4). The capillary portion is to be converted into needles or hairs by drawing out in a microflame (Fig. 1). The shape of the needle can be greatly varied. Factors that influence the result are the hardness and the diameter of the capillary used, the size of the flame, the

<sup>1</sup> Spemann, H. *Abderhaldens Handbuch d. biol. Arbeitsmethoden*, 5: 3, 1923.

<sup>2</sup> Mangold, O. *Péterfis Methodik der wiss. Biol.*, 1928, vol. 2.

<sup>3</sup> Zoja, R. *Arch. Entw.-mech.*, vol. 2: 1895.

<sup>4</sup> Delage, Y. *Arch. Exp. Zool.*, s. 3, vol. 7: 1899.

<sup>5</sup> Spemann, H. *Verh. d. deutsch. Zool. Ges.*, 1906.

strength, direction and time of the pull. One soon learns how fast to pull in order to get a long, fine thread or a short, sharp point; or how to make a bent hair by an oblique movement with one hand during the pulling. When a service-

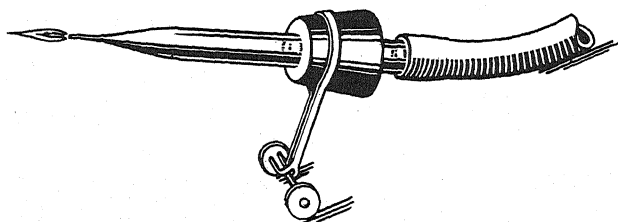


FIG. 1. Microflame. After Spemann.<sup>1</sup>

able point has been obtained, a length of the capillary is broken off about 3 cm. from the needle tip and fastened to the handle by melting in the microflame. As soon as the two pieces stick together, the joint is brought again into the flame with the glass held vertically, the needle hanging downward. As the joint melts it becomes converted into a ball which shortens the capillary and strengthens the seal (Fig. 4).

Spemann recommends the following way of making the needles:

The end of the long glass thread, still attached to the handle, is provided with a small hook by heating the thread for a moment in the microflame. The thread with its handle down is now hung to a support by means of the hook.

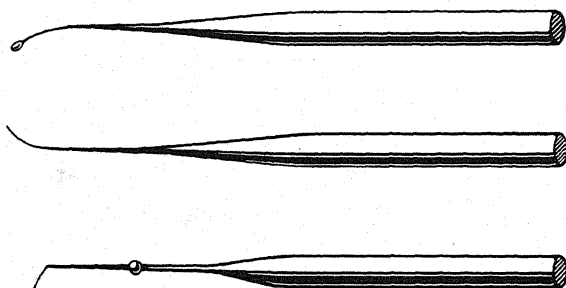


FIG. 2. Hairloop. FIGS. 3 and 4. Glass-needles. After Spemann.<sup>1</sup>

The thread, at a convenient distance from the handle (about 3.4 cm.) is heated in the microflame until it stretches and separates, the handle dropping into a cylinder with cotton on the bottom. The straight glass hair obtained in this way can afterwards be bent at the desired angle by approaching it to the microflame or by leaning it against a piece of heated metal.

As the microflame often becomes extinguished it is useful to provide the gas tube with a Y-tube and have two flames: one a larger flame to serve as a pilot light for the other, the microflame, the tubing of which should have a Hoffman screw clamp for regulating the gas pressure.

A Spemann hair loop (Fig. 2) is a good instrument to move or hold delicate objects such as young stages of Amphibian eggs which have been removed from their membranes.

A human hair is looped and the two ends inserted into the capillary end of a hollow glass tube made like one of the handles already mentioned. The ends of the hair are pushed in until the loop reaches the desired size. The loop is then fixed in place by applying melted wax on a heated scalpel to the mouth of the capillary. The wax runs into the tube and, on cooling, fastens the hair.

To cut out small pieces of tissue for implantation, Spemann originally used a glass pipette (Fig. 5) with a rubber nipple on one end and with a small opening on one side, covered with a piece of rubber tubing. By gently releasing the pressure on this rubber membrane, a minute piece of tissue could be drawn into the pipette and then isolated by cutting across the mouth of the pipette with a fine knife. This isolated piece could then be placed in a cavity in another individual produced by cutting out a piece in the afore-mentioned way. At present, in place of Spemann's pipette most transplantations on Amphibians are made by means of the hairloop and glass needles or steel knives, the needles or knives being used for cutting. For cutting out transplants in older Amphibian embryos, Harrison uses a pair of embryological scissors. The glass needles for work on Amphibians must be comparatively thick, with short, sharp points, bent at some distance from the point (5-8 mm.), by nearing it to the microflame. The cutting operation with such a needle is performed by pressing with the bent end more or less parallel to the surface of the object.

For the separation of blastomeres of eggs smaller than Amphibian, it is advisable to have a long, fine needle, a glass hair, slightly curved near the point (Figs. 3, 4). The curved end is applied to the bottom of the dish containing the eggs. This steadies the needle and the cut is made by pressing upon the egg with the shaft of the glass hair between the curved bend and the handle.

To move very small eggs or blastomeres from one dish to another, a mouth pipette is extremely useful (Péterfi, 1924<sup>6</sup>; Fry, 1924<sup>7</sup>).

A fairly long pipette is drawn out in the microflame, near its end, so that the capillary ends with a point. With a diamond a slight mark is made on that part of this pointed capillary which has a little larger inner diameter than the eggs or larvae in question; and the capillary is then broken here. The pipette is connected with a rubber tube, to the other end of which a short glass tube as mouth-piece may be attached.

<sup>6</sup> Péterfi, T. *Abderhaldens Handb. d. biol. Arbeitsmethoden*, 5: 2, 1924.

<sup>7</sup> Fry, H. *Anat. Rec.*, vol. 28: 1924.

This is fixed on the dissecting microscope at a height convenient for the lips to reach, with the pipette laid in a score made in the side of a box. The pipette is then filled—by capillarity, when it is ready for use. By gently aspirating, the desired object is sucked into the pipette. One advantage of this pipette is that it can be laid down on the table, leaning on the support mentioned, with the point directed obliquely upwards. When dishes are changed, the object remains safely close to the mouth of the pipette. Another advantage is that one can handle the pipette much more steadily since it is not necessary to regulate the pressure with the fingers. Some training is required in order not to suck or blow too fast.

A suitable surface for placing the eggs is essential for the operation. Many small marine eggs can be cut on a glass bottom. Some workers orient and cut the eggs in a drop of water on a clean, grease-free slip, after pushing it near the edge of the drop, where the decreasing depth of water slightly flattens and holds it. The following method (Hörstadius, 1928<sup>8</sup>) has many advantages. On the bottom of a shallow dish is placed a thin disc of celluloid, e.g. a piece of well-washed (warm water) photographic film, in which slight grooves have been made by scratching it with a pin. The egg, which is likely to glide away from under the needle when on a glass bottom, is easy to cut when it lies in the groove. The depth, breadth, and surface (rough or smooth) must be varied according to the type of cell which is to be cut.

For Amphibian eggs Spemann recommends a working surface of wax (black or white). Agar-agar, gelatin and cellophane may also be used. Schotté (1930<sup>9</sup>) suggests silk for the delicate Anuran eggs. He stretches the silk over a glass ring, which is then placed on the bottom of the operation dish. Other workers place the silk directly on the bottom of a Syracuse watch glass. An advantage of wax or agar-agar is that depressions can be made in which to place the objects. Moreover, many invertebrate eggs adhere to the glass bottom, in which case they do not develop. Some eggs do better on celluloid, others on agar.

Most eggs cannot be operated on until the surrounding jelly and the fertilization membrane have been removed. In many invertebrate eggs this can be done simply by the old method of shaking the eggs in a vial half filled with seawater (Driesch 1893<sup>10</sup>), or by sucking them into a pipette (Plough, 1927<sup>11</sup>), the bore of which has about the same diameter as the egg in question. The membranes of Amphibian eggs have to be removed carefully with two well-ground watchmaker's forceps. Dalcq

<sup>8</sup> Hörstadius, S. *Acta Zool.*, vol. 9: 1928.

<sup>9</sup> Schotté, O. *Roux's Arch. Entw.-mech.*, vol. 123: 1930.

<sup>10</sup> Driesch, H. *Anat. Anz.*, vol. 8: 1893.

<sup>11</sup> Plough, H. *Biol. Bull.*, vol. 52: 1927.

(personal communication) has used this method on such small eggs as those of Ascidians. A window can be cut in the hard membrane of the fish egg with a pair of embryological scissors. In order to prevent the

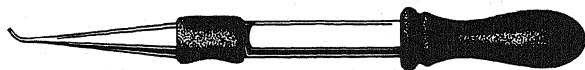


FIG. 5. Pipette for microtransplantation. After Spemann.<sup>1</sup>

point of the scissors from gliding out of position under the membrane, Nicholas<sup>12</sup> recommends having the upper blade shorter than the under one.

In order to separate blastomeres of marine eggs, it is often serviceable to perform the operation in calcium-free sea water, which loosens the connections between the cells.

The original formula by Herbst (1900)<sup>13</sup> is the following: 96.2 per cent aq. dest., 3.07 per cent NaCl, .08 per cent HCl, .66 per cent MgSO<sub>4</sub>, MgHPO<sub>4</sub> in excess. Another formula is that of Bialascewicz and Runnström (see Hörstadius, 1935<sup>14</sup>) (salinity, 3.5 per cent):

3.29 per cent NaCl .....	100.0 c.c.
4.28 per cent KCl .....	2.0 c.c.
3.49 per cent MgCl <sub>2</sub> .....	10.5 c.c.
19.50 per cent MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	3.0 c.c.
5.21 per cent NaHCO <sub>3</sub> .....	0.1 c.c.
2.6 per cent NaOH .....	0.1 c.c.

A calcium-free sea water (by Moore), used at Woods Hole, is prepared as follows: NaCl 19.07 gm., KCl 0.54 gm., MgCl<sub>2</sub> (6 H<sub>2</sub>O) 5.18 gm., MgSO<sub>4</sub> (7 H<sub>2</sub>O) 3.07 gm., H<sub>2</sub>O to 1,000 c.c.

The eggs are brought into the calcium-free sea water about half an hour before operation and brought back into sea water immediately after operation. Not all kinds of eggs are susceptible to this treatment.

It is not appropriate to operate upon the Amphibian egg in fresh water. Ringer's solution may be used, but recently the following solution, suggested by Holtfreter,<sup>15</sup> has come most into use: 0.35 gm. NaCl, 0.005 gm. KCl, 0.01 gm. CaCl<sub>2</sub>, NaHCO<sub>3</sub> 0.02 gm., H<sub>2</sub>O 100 c.c. The bicarbonate has been found to be unnecessary and may be omitted, especially since it tends to produce a precipitate on sterilization.

Operations in sea water are generally made without sterile precautions. The composition of sea water is changed by heating, but it may be sterilized by passing it through a Berkefeld filter. A sterile technique

<sup>12</sup> Nicholas, J. S. *Proc. Nat. Acad. Sc.*, vol. 13: 1927.

<sup>13</sup> Herbst, C. *Arch. Entw.-mech.*, vol. 9: 1900.

<sup>14</sup> Hörstadius, S. *Pubbl. Staz. Zool. Napoli*, vol. 14: 1935.

<sup>15</sup> Holtfreter, J. *Roux's Arch. Entw.-mech.*, vol. 124: 1931.



during operations, first suggested by Woerdemann,<sup>16</sup> has greatly decreased the mortality of Amphibian eggs. All dishes, covers and solutions to be used are sterilized in an autoclave, the solutions being in bottles with

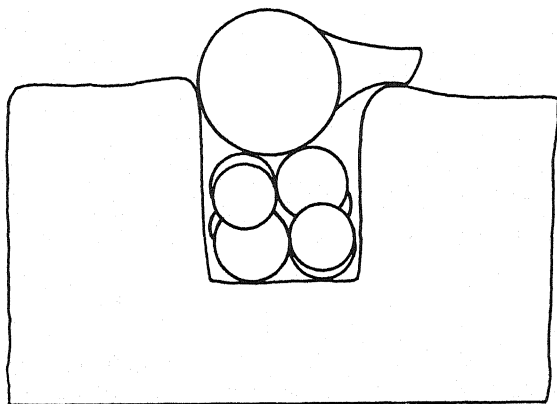


Fig. 6. Transplantation of blastomeres. After Hörstadius.<sup>8</sup>

cotton stoppers and not too full and the glassware wrapped in paper. The eggs are removed from the jelly and the capsules and the vitelline membrane left intact. They are then washed by passing through eight successive changes of sterile water. The mouth of the water bottle should always be heated in a Bunsen flame immediately after removal of the cotton stopper, and the water poured into 8 sterile Stender dishes, the covers raised only enough for pouring. The eggs, still in their vitelline membranes, are brought into the first dish with a pipette which has previously been sterilized by flaming. The eggs are rotated in the dish and then transferred from one dish to the other, each time with a fresh sterile pipette. After the eighth washing they are brought into a dish containing Holtfreter's solution, where they remain until the time of operation. They are now transferred with a sterile pipette into the sterile operation dish (Syracuse watch glass), where the vitelline membrane is removed. The instruments (forceps, knives, scissors, glass needles, hair loops) are kept in a dish with strong alcohol and only taken out just before the operation. It is preferable to have two sets of instruments, one of which is being sterilized in alcohol while the other is in use. After the operation it is advisable to move the dishes as little as possible. After some days the embryos are transferred to water. The delicacy of the operative technique and the sterile precautions required make imperative the avoidance of draughts, etc., and any disturbing influences. Later

<sup>16</sup> Woerdemann, M. W. *Roux's Arch. Entw.-mech.*, vol. 121: 1930.

embryo stages of the Amphibia are not so susceptible to infections, so that they can be operated on without sterile technique.

To get an Amphibian egg implant to adhere it is often necessary to apply some pressure for a short time. For this purpose small bridges of glass are used. They are made of a fine glass thread or of a narrow piece of a cover-slip cut with a diamond and then bent in the desired manner in the microflame, or by heating them on a piece of metal of appropriate form. For transplantation of blastomeres of very minute (Echinoderm) eggs, Hörstadius<sup>8</sup> suggests the following method: In the photographic film (cf. p. 46), on the floor of a dish, a depression is made by rotating a pin, the point of which has been made blunt by pressing against glass or metal. The two pieces of the segmented egg that are to be joined are lowered into the depression in the right orientation, one on top of the other (Fig. 6). To supply pressure a small ball of glass is placed over the depression. The balls (see Fig. 6) are made in the following way: A capillary (see foregoing) is heated in the microflame and then very quickly drawn out to a very fine thread. The end of this thread is heated in the microflame till it forms a ball which is broken off with fine forceps under water. The balls covering the objects in the depressions must be removed fairly soon in order not to cause any malformations. A quarter of an hour to half an hour is soon enough.

In order to recognize the transplant in its further development, Harrison (1904)<sup>17</sup> and Spemann<sup>1</sup> have used different species having unlike color and cell size as host and donor. Detwiler<sup>18</sup> implanted pieces from embryos that had previously been vitally stained. In the sea-urchin larvae Hörstadius<sup>8, 14</sup> cut out a group of blastomeres, brought them for a couple of minutes into a weak solution of Nile blue sulphate, washed them in another dish of sea water, and transplanted them back into their original position in the egg. The important method of local vital staining, through the diffusion of dye adsorbed on small pieces of agar, was worked out by W. Vogt (1925).<sup>19</sup>

A solution of agar is poured out in a flat dish and dried so that it forms a thin film, pieces of which are stained in a 1 per cent solution of Nile blue sulphate, Neutral red or Bismarck brown. The agar is then washed until it does not give off clouds of stain into the water. When small pieces of this agar are applied against an embryo, the stain will diffuse into the tissues, giving a stained mark of the same size and shape as the piece of agar used. To bring the pieces of agar into contact with the Amphibian egg, Vogt makes a depression in wax with a glass needle which has been melted in the microflame to a ball of the same size as the egg. In the bottom of this depression small cavities are made with a fine

<sup>17</sup> Harrison, R. G. *Arch. mikr. Anat.*, vol. 63: 1904.

<sup>18</sup> Detwiler, S. R. *Anat. Rec.*, vol. 13: 1917.

<sup>19</sup> Vogt, W. *Roux's Arch. Entw.-mech.*, vol. 126: 1925.

needle and small pieces of the agar are placed in them with the aid of the hair loop. The egg, placed in the depression, will thus get a series of marks on its under side (Fig. 7). In order to obtain stained spots also on the lateral and upper

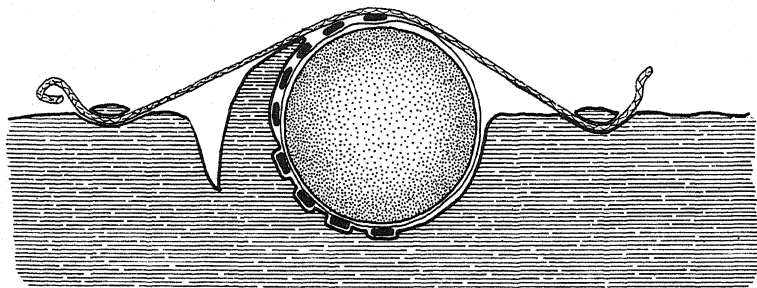


FIG. 7. Local vital staining of an Amphibian egg. After Vogt.<sup>19</sup>

sides, a part of the wax is pressed up against the egg, stained pieces of agar inserted between the egg and the wax, and other pieces can be laid under a bridge of tinfoil placed over the egg (Fig. 7).

A blastomere of a very small egg can be stained by leaning it against a small piece of agar on the bottom of a glass dish (v. Ubisch, 1925).<sup>20</sup> The segmented egg is removed when the stain has penetrated about three-fourths of the blastomere in question. The stain will later spread through the entire cell, but will not diffuse into the adjacent cells if the staining has not been too strong. It is important that no clouds of stain be given off into the water by the stained pieces. For setting very small marks ( $5-10\ \mu$ ) on very minute objects, e.g. blastomeres of a later cleavage stage, Lindahl<sup>21</sup> draws out a capillary tube into a still finer and short capillary ( $5-10\ \mu$  in diameter) and breaks it off just where it narrows, by mere pulling, outside the flame, so that one gets a fine, smooth pipette mouth of the size desired. The capillary is broken off about 5 cm. from the fine mouth and this end closed by melting. Such capillaries with only one, very fine opening, are put into a warm solution of 1 per cent agar in sea water with 1 per cent Nile blue. As cooling takes place, some of the solution will be sucked into this micropipette. The capillaries are left in the agar up to the moment of use, when they are washed off. It is important not to keep them too long in the air, as the agar may withdraw from the mouth. Unless one has a steady hand it is advisable to insert this capillary pipette into an ordinary pipette (bent a couple of centimeters from the mouth to prevent the capillary from gliding too far in) and then, using the latter as a holder, to fix it in a micromanipulator before applying the egg against the opening with the agar.

<sup>20</sup> v. Ubisch, L. *Z. wiss. Zool.* 124: 1925.

<sup>21</sup> Lindahl, P. E. *Roux's Arch. Entw.-mech.*, vol. 127: 1932.

## FREE-HAND OR PARTLY MECHANICAL METHODS OF MICROINJECTION WITH WIDE RANGE OF CONTROL

H. McE. KNOWER

Under the heading microinjection will be included procedures which can be carried on and controlled while being watched under the binocular microscope. There is no fundamental difference between the grosser methods of injection and those included in this section, though the latter naturally require more delicate adjustments with the end pipette, pressure, etc.

Perhaps the most available general method for use with either gross material or small specimens injected under the microscope is that of H. Hoyer<sup>1</sup> also described in *Enzyklopädie der mikroskopischen Technik*<sup>2</sup> and by Mozejko.<sup>3</sup> This technique depends on an ingenious method of controlling the pressure of injection through a specially devised metal valve operated by the foot. The valve is introduced in a line of tubing which reaches the injection table from a cylinder of compressed gas. In addition to these special features it is necessary, if the procedure is one of microinjection, to be provided with a sufficiently fine micropipette at the end of the tubing. This apparatus should be kept at hand assembled on a special table, and combines several important advantages: (1) the facility with which one may inject either relatively large specimens or small embryos; (2) the ready control of injection while being watched under the microscope, and (3) a very important feature—the use of the foot pedal, which leaves both hands free for the operation. The many important publications from Hoyer's laboratory at Krakau, Poland,<sup>4</sup> witness the value of this technique with a wide range of specimens.

The methods to be grouped especially under the heading "Microinjection" may be divided into free-hand, and more or less mechanically controlled procedures.

1. **Free Hand.** H. M. Evans published a number of papers in 1909<sup>5</sup> and a chapter in the Keibel-Mall "Human Embryology" (1912) presenting the first comprehensive account of a series of complete capillary injections with full details. These accounts are concerned with injections of

<sup>1</sup> Hoyer, H. *Ztschr. f. wiss. Mikrosk.*, vol. 25: 1908.

<sup>2</sup> Krause, R. *Enzykl. der mikr. Tech.*, 2:1104, 1926.

<sup>3</sup> Mozejko. *Ztschr. f. wiss. Mikrosk.*, vol. 28: 1911.

<sup>4</sup> *Bull. de l'Acad. des sc. Polonaise de Cracovie*, sér. B; also Memoirs of this Academy by Hoyer and Grodzinski on comparative anatomy of lymphatics and blood vessels.

<sup>5</sup> Evans, H. M. *Am. J. Anat.*, 1909; *Anat. Rec.*, 1909; Keibel-Mall: *Human Embryology*, 1912.

young chick and mammalian embryos. The method used by Evans was adapted from that of Popoff (1894), a free-hand method of great value and general usefulness. In this method a tube of soft glass 2 to 3 mm. in

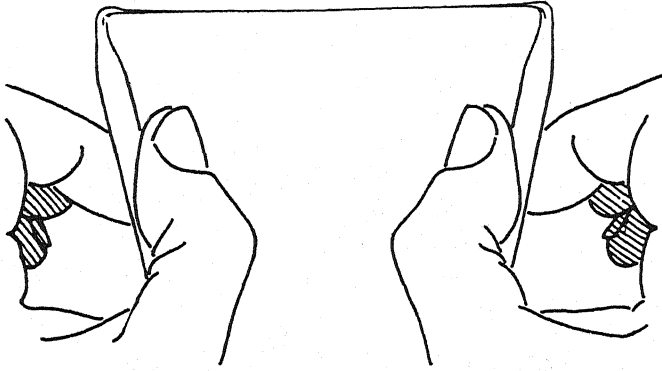


FIG. 1. Method of drawing the micropipette for free-hand technique.

diameter is drawn out at the ends, a suitable tip being secured, after bending the drawn capillary at right angles, by re-drawing the tip through the margin of a microflame to produce a reduced patent filament of the desired fraction of a millimeter in diameter (see Summary, Section 4, p. 59). The pipette, filled with injection fluid, is attached by its larger end to a rubber tube which is held in the mouth, the injection pressure being obtained by blowing into the tube. A most important feature of this technique, as with others, is the fine calibre of the tip of the pipette, which should narrow rapidly from the main tube to give least capillary resistance.

Though never as fine as those used by Chambers in micrurgical work with high powers of the microscope, a similar method is used for drawing the tip of these pipettes. (See Summary, section 4, p. 59; also Dr. Chambers' section, p. 86.)

In this connection the method of drawing the capillary tube used by the Clarks<sup>6</sup> is of value for insuring the production of a point sufficiently fine, yet reducing the capillary resistance by the rapid narrowing of the tube in approaching the tip.

To produce such a pipette, a relatively soft glass tube of about 2 mm. in diameter should be rotated in the flame while held with the hands pronated. As soon as the glass between the hands becomes sufficiently heated, the thumbs are pressed firmly on the tube against the index fingers, on either side of the flame, and the hands are pushed away from the body while the thumbs are at the same time turned laterally right and left and rapidly separated from each other.

<sup>6</sup> Clark, E. R. and E. L. Lymphatics. *Carnegie Inst. Contr. to Embryol.*, No. 272, 1920.

This results in a right-angled bend at the heated end of the pipette, with a fine filiform tip beginning immediately at the bend and a maximal reduction of capillary friction (Fig. 1). (For re-drawing a finer tip, see Summary, Section 4.)

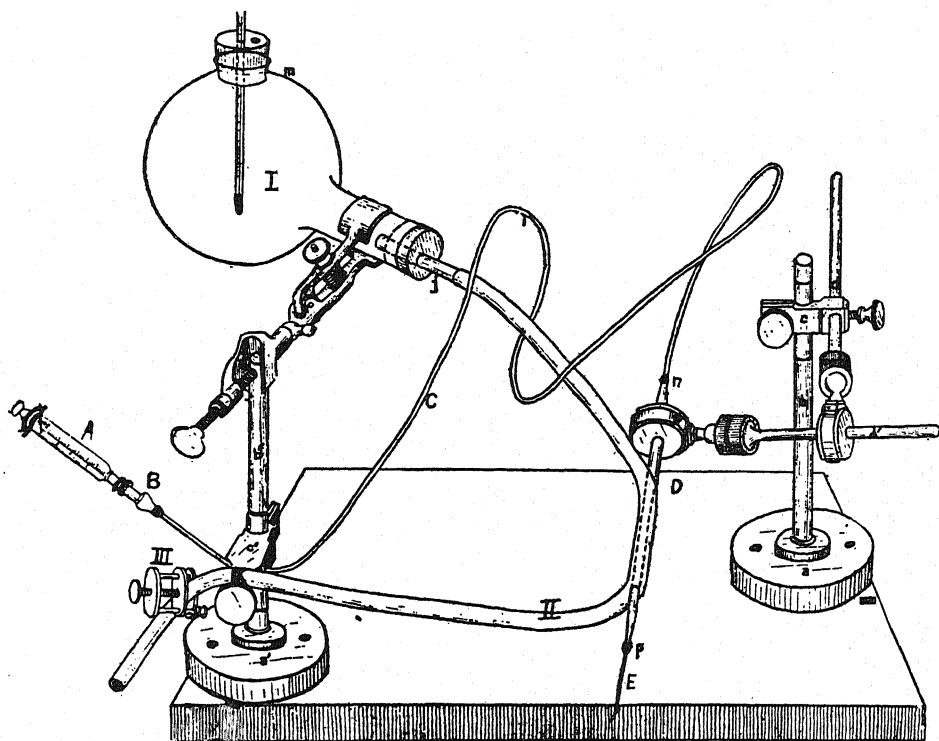


FIG. 2. Brown's method for making cold or warm injections into small blood vessels. For description see text.

This very valuable free-hand method has been used by many investigators for both blood vessels and lymphatics (see papers by H. M. Evans,<sup>5</sup> F. R. Sabin,<sup>7</sup> and the Clarks<sup>6</sup>). The procedure is of general usefulness and especially adaptable to injections of chick and mammalian embryos. The arrangement of the specimens and various steps in injecting are described under appropriate headings later (see Summary of precautions and suggestions).

**2. Partly Mechanical Methods with Wide-range Control.** Though the foregoing method is available for use with most embryos in the hands

<sup>7</sup> Sabin, F. R. Lymphatics. (A series of important revolutionary articles on the lymphatics including the technique of injecting.) *Am. J. Anat.*, 1901, 1904, 1905, 1909; *Anat. Rec.*, 1908, 1911, 1912; and Keibel-Mall: *Human Embryology*, 1912.

Blood vessels. *Carnegie Inst. Contr. Embryol.*, no. 223, 1915; and no. 226, 1917.

of an operator with necessary skill, a more controlled or partly mechanical method of manipulation and securing pressure seems preferable for certain materials and for the average operator. Of such methods, special mention should be made of Chambers' microinjection apparatus adapted by Brown<sup>8</sup> for injecting the blood vessels of chick embryos under the microscope (Fig. 2).

The apparatus (Fig. 2) consists of an ordinary 2 c.c. glass syringe, A, which fits into the adapter, B, of a hypodermic needle, whose tip is fastened with shellac or de Khotinsky cement into one end of a long, coiled piece of slender, soft, brass tubing, C, into the other end of which is cemented at N, a four inch piece of  $\frac{1}{8}$  inch glass tubing, D. The glass tube is drawn out at its free end into a slender shank with a bore having a diameter of about 1 mm., into which the injection cannula, E, is to be sealed with de Khotinsky cement. The cannula is a glass capillary, the tip of which is made by momentarily heating the capillary over a microburner and pulling it out just as it softens. The tapering tip may be broken off to form a pipette of the desired size. The support for the injecting device is a ball-and-socket contrivance which may be built of two Leitz dissecting stands.

A warm injection can be accomplished by arranging a flow of warm water to heat the injection fluid in the glass tube which carries the cannula. The glass tube is thrust through the wall of a  $\frac{1}{2}$  inch rubber tubing, II, in two places. The upper end of the rubber tube is connected with a flask of hot water, I, and the lower end, which serves for the outflow, is shunted off to one side with a screw clamp, III, to regulate the flow.

*For work under very high power of the microscope, Chambers' special methods of microinjection, described on page 86, are to be recommended.*

Another partly mechanical method devised by Knower (1908)<sup>9</sup> obtains the pressure for injection from the expansion of air in a closed system in a glass bulb on the end of a capillary tube. Preliminary to injecting, the fine point of the tube on the end of which the bulb is blown, is held beneath the surface of some fluid (as India ink or Berlin blue) and air is driven out of the bulb by warming; the fluid then running up to replace the air as the bulb cools. When the system has come to an equilibrium the point of the tube can be inserted into the selected vessel under a binocular microscope, the necessary heat applied, and the injection performed. (See Fig. 3.)

A few directions should suffice to make this method readily available.

A tube of not too hard glass from 2 to 4 mm. in diameter is heated in the edge of a Bunsen burner, and rotated while heating, until it can be drawn out rapidly

<sup>8</sup> Brown, A. L. (Chambers). *Anat. Rec.*, 24: 295, 1922.

<sup>9</sup> Knower, H. McE. *Anat. Rec.*, 2: 207, 1908.

as in Figure 3 (1). The capillary is then broken across and one of the heavy ends heated at the point *x* in Figure 3 (2) to burn off the superfluous tubing and leave a mass of glass on the end of the capillary. The enlarged mass at the

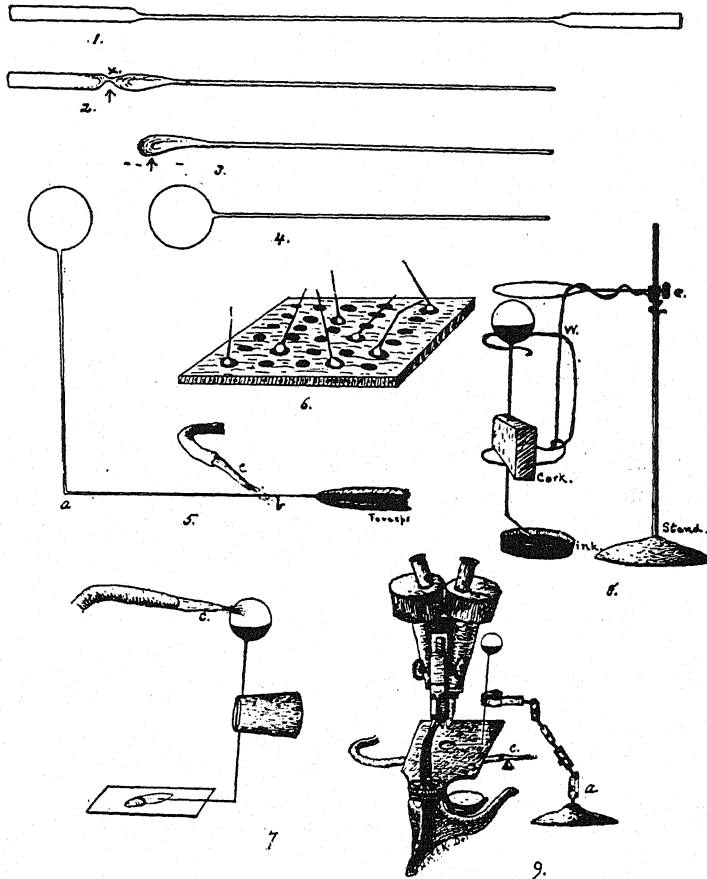


FIG. 3. Knower's method for making capillary glass bulbs and adjustments for injecting small blood vessels. For description see text.

end of the capillary is now turned head-on into the margin of the flame and heated again until molten to the point of sagging. Avoid melting the adjacent capillary tube. At the favorable moment the end is removed from the flame and blown out quickly, but without too great pressure, through the capillary tube (Fig. 3, No. 4). The size of the bulb is usually  $\frac{1}{2}$  inch or less in diameter; and for ordinary use a thickish wall is preferable, though in certain cases greater sensitiveness may be secured by a thinner wall. A right-angled bend is now given the capillary as in Figure 3 (5) by holding it in the margin of a microburner, and following this, a tip of the desired fineness is given to the end by again



drawing the capillary through the periphery of the microburner, turned down to a minimum blue point of flame. The flame appears too large in Figure 3 (5). (See Summary, Section 4, p. 59, for the exact method of securing this fine tip.)

It is important to obtain a very fine point to insure good results even when piercing the wall of a large vessel. Most difficulties which arise are to be traced to insufficient fineness of the tip. The injection bulbs may stand on a board as in Figure 3 (6), or, if not too thin, in an ordinary glass tumbler or Stender dish. A suitable holder for the injector is very important in this technique, and the type pictured in Figure 3 (9) is believed to be the most successful. This is a jointed arm formerly used as a lens holder, now furnished by a number of dealers as a support for microlamps (for instance the Spencer Universal Lamp no. 358 is supported by such a holder). Having tried a variety of devices for supporting the injector, we consider that this assembly of ball-bearing joints is to be recommended, since, when properly adjusted, the tip of the injector will stay in the position in which it is placed. Other arrangements of bent metal rods (lead, etc.) or metal tubing are subject to vibration or recoil, springing back from the point to be injected. With the ball-bearing holder, as soon as the tip is secured in the desired position, the holder of itself becomes fixed at once and permits the injector to remain in place without jerks or recoil, while the hands are free to aid in various manipulations.

Another type of holder used by Earl Perkins<sup>10</sup> with small Crustacea is an improvised column of modelers' "plasticine" clay. The cork, with the injector in place, can be stuck into the top of the column of clay and used as with the ball-and-socket holder, since the plasticine will go into action without vibration or recoil.

Preparatory to injecting, the capillary tube of the bulb is wedged into the end of a cork, split with a safety razor (Fig. 3, No. 7). The cork should be trimmed square to fit the holder as in Figure 3 (9). To fill the bulb, any support which will hold the tip just beneath the surface of the injecting fluid without breaking it will serve. A rack may be constructed as in Figure 3 (8), or it may suffice to rest the cork on the side of a dish, allowing the tip of the tube to hang down into the fluid. If the injector is fixed in the holder described above, this will keep the tip in position ready for use.

In advance of injection the bulb is warmed and allowed to fill with the injecting fluid, and when an equilibrium is secured the holder, with the bulb in place, is brought over the table of the dissecting microscope as in Figure 3 (9a) and a microburner on a flexible, light-weight rubber tube placed in front at (c). Two or more injectors should stand loaded at the right hand with tips in fluid.

<sup>10</sup> Perkins, E. B., and Brody, M. S. *J. Morph. & Physiol.*, 50: 129, 1930.

When the tip has been successfully entered, under the binocular microscope, into the location selected in the specimen, the bulb is heated gently with the left hand by a flick from the small microburner, as indicated in Figure 3 (7). A single flick of the bulb may be sufficient for injection of a limited area, but usually the heating must be repeated (see Summary Section 6, p. 60). Though the pressure rises quickly, the capillarity of the tube reduces the effect so that the pressure need not get out of control and may be varied at will. When not in use, the gas jet lies ready in front of the operator and the tip of the injector, after a quick cleansing with the left fingers dipped in water, is immersed in fluid and allowed to refill.

This method has been found most adaptable to use with small fish embryos (trout, toadfish, dogfish, etc., young larvae of various Amphibia, and other small animals). Its advantages with such material are the ease with which specimens are made ready, injected and removed, to be replaced by others; while the injectors, supported on holders, may be quickly substituted at will, one for another, in single or double injections. Other advantages are:

1. Less manipulative skill is required than with those methods depending on pressure from a column of fluid, a rubber bulb, a syringe or from the mouth.
2. No rubber tubes or other appendages interfere with free movement.
3. With the use of the jointed holder the maximum effort may be devoted to introducing the point into a vessel, while a minimum of attention is then demanded to applying the warmth, or other manipulation.
4. Pressure is variable at will, and especially sensitively controllable.
5. Fluid does not run out until desired, if a fine point is used.
6. The amount of fluid is easily measured, so that a quantity as small as needed may be introduced into a vessel of a limited area.
7. The bulbs hold fluid well without evaporation, and the quantity is seen at a glance through the glass.
8. The apparatus is simple and easily made.
9. The tubes are very adaptable in size and shape to special conditions.
10. The tips are readily made of any degree of fineness.

### 3. Summary of Precautions and Suggestions for Microinjection.

The following suggestions and precautions will be found useful, whatever method of microinjection is used.

1. Material and methods adapted to different types:

The general method of Hoyer is adaptable to all types of specimens, large or small.

With embryos of lizards, birds or mammals, use methods of Evans, with various modifications, or Brown-Chambers.

With small embryos of fish, Amphibian larvae, and all very small animals, use, preferably, the method of Knowler; or use Evans' method.

Whenever possible, select animals with a minimum of pigment or with pigment which washes out in alcohols after fixation.

Albuminous fluids and membranes should be removed from embryos before injecting.

Salt water specimens should be washed off in fresh water immediately before injecting, as the sea water tends to coagulate the ink of the injecting fluid.

### 2. Preparation of specimen:

Embryos should be disposed on the injecting table according to their character (lighting, choice of power of the microscope and of vessel is described in Sections 6 and 7 of this Summary).

Chick embryos to be injected by the Evans-Popoff method, or a modification of it, can be handled in the egg shell in situ after the albumen and surface membrane have been removed; or the entire blastoderm may be removed intact to a warm slide. The left hand should exert a gentle traction, against the push of the right, through fine forceps fixed on tissues around the distal end of the vessel injected. Permit the heart beat to help the process.

In a similar way the umbilical cord of small mammalian embryos or shark embryos may be pierced by the injecting tip with the injector bulb in the right hand. It is often well to anchor the distal end of the cord by a tractor or weight of some sort.

Animals from sea-water should be washed off in fresh water as sea water coagulates the ink of the injecting fluid.

Most small embryos should be furnished with a bed or trough built of paraffin on a glass slide, with low walls open at the right to permit the specimen to be slipped in, with some part of the body, as the tail or leg, projecting. Mammalian embryos may be kept warm in body fluids in this way. Shark embryos can be kept in normal salt solution, while fish embryos and Amphibian larvae can be also kept in position in one of the beds described, with the tail or leg accessible, lying flat on the glass surface. The bed or trough not only helps to keep the specimen moist, but serves as a useful resistance to the push of the pipette from the right side.

### 3. Anesthetizing:

It is not always necessary to anesthetize the embryo. If advisable, chlorotone is one of the best substances to use. Care should be taken to avoid checking the heart beat more than necessary. Chlorotone should be used in any event just before fixing to inhibit movements.

#### 4. Preparation of pipettes:

Do not use glass tubing of too hard a consistency.

First draw out into fine tubes of a diameter somewhat greater than that to be used at the tip, which should be secured by re-drawing in the edge of the flame of a microburner turned down to a minimum blue point of flame. It appears too large in Figure 3 (5). Draw toward the body through the periphery of the microflame.

Usually bulbs should not be too thin-walled, nor very large; though large, thin-walled bulbs responding to the mere warmth of the hand are good for injection of small areas or special conditions (Perkins).<sup>10</sup>

Emphasis should be repeated on the importance of a very fine, sharp tip to be kept freely open at all times by immersion in water or other fluid when not in use.

It is also important to keep the length of the capillary tube down to the minimum to reduce friction of wall.

Preparatory to injection two or more pipettes should be loaded and placed near the right hand, with the tips in fluid.

#### 5. Injecting fluid:

Most workers have found India ink (Higgins) one of the best fluids for injection, either full strength or often, as Evans pointed out, diluted with distilled water. A suspension of "insoluble" Berlin blue in water is another very delicate and desirable material.

Excellent double injections may be made: first the Berlin blue, then India ink, or the fluids in reverse order.

Silver nitrate after Sabin's technique is another useful fluid. (See Footnote 7, page 53.)

Other fine powders in solution or suspension are also to be recommended.

#### 6. Introduction of the tip of the injector:

This operation should be observed and the injection carried out in a strong light from above through a condenser and under an intermediate power of the binocular microscope; with high enough magnification to permit easy identification of arteries and veins by means of the blood circulating in them, yet of sufficiently wide field to enable the spread of the injection to be watched in different regions at the same time. High powers give such a small field as to prevent adequately watching the progress of the operation, hence are advised only for limited local areas.

Two or more loaded injectors should stand near the right hand, tips in fluid.

As pointed out by Evans it is generally best to select an artery for entering the system, though veins are often used; vitelline arteries in bird embryos or young fish; umbilical artery in mammals, shark embryos, etc.; caudal arteries in fish, young Amphibia; brachial arteries in older Amphibia.

Lymphatic injections should be made through one of the main trunks which

can be entered from branches in the tissues surrounding certain veins seen in the field; in fish embryos, accordingly, the injector tip should pierce the tissues along the vein of the lateral line, or in the tail ventrally, next to the caudal vein. In young Amphibian larvae aim to enter a caudal trunk, either ventral to the caudal vein, or a lymphatic, dorsal to the spinal cord. The lateral line lymphatic offers another alternative in Amphibia, especially in older larvae with hind limbs, and posterior lymph hearts are thus easily demonstrated and watched while in action. In these older specimens most of the lymphatic system can be injected from the lymphatics of the legs.

In approaching the selected vessel with the tip of the injector the left hand helps in manipulation of the specimen, while the right introduces the pipette into the desired small vessel. Thus, in the case of the chick, the left hand can dispose of the membranes and create a slight tension by forceps on the vitelline vessels. In the mammal or shark the umbilical vessels can likewise be rendered more approachable by the use of this hand.

As the tip is seen to reach the point selected for piercing the vessel wall, slight pressure is brought to bear on the fluid in the injector and a few drops forced from the tip, the result being merely a deposit in the connective tissue unless a vessel wall is pierced. Failing to inject, the tip should be pushed further in, along the selected vessel, and pressure again applied to the injecting fluid when the wall appears to be pierced. Soon the wall will be broken and entered by the injection, which immediately brings the vessel and its branches into view and spreads far beyond as pressure is maintained and increased.

In injecting specimens which have slimy, scaly or tough surfaces, it is often advisable to first penetrate the surface by means of a very fine needle point and then follow in with the point of the injector. In certain cases of larger embryos, it is possible to cut through the surface tissues to a considerable depth in order to expose a deep artery (for instance, branchial vessels of large Amphibia may be exposed by cutting away the ventral portion of the operculum and the caudal artery may be reached by cutting away muscles). The injection may then be made without serious loss of blood in regions beyond the cut.

Heuser<sup>11</sup> secured beautiful results in the branchial regions by injecting directly into the heart of certain mammalian embryos while watching the distribution of the fluid.

Perkins<sup>10</sup> was also aided by heart action in injecting small crustacea, using glass bulb injectors with very thin walls warmed by the fingers.

#### 7. Lighting:

A strong light from above should be directed on the operating table through condenser lenses. This is of primary importance, though it may be supplemented from below. The same light is advised for cleared specimens.

<sup>11</sup> Heuser, C. H. *Carnegie Inst. Contr. to Embryol.*, no. 332, 1923.

### 8. Fixation:

Formalin can be used to fix most embryos satisfactorily; but sharper results are secured by immediate immersion in corrosive sublimate plus 5 per cent acetic acid ( $\text{HgCl}_2$  + 5 per cent acetic); or in Bouin's or Carnoy's fluid. With young Amphibian larvae, mercuric-acetic has been found to be most satisfactory, and such specimens should be washed for several days in 70 per cent alcohol with a little iodine added. This not only removes the mercury but also helps to remove pigment in various species.

### 9. Dehydration and clearing:

Thorough dehydration followed by clearing with cedar-wood oil is advised, and varying amounts of methyl salicylate may be added to the cedar-wood oil to give more or less prominence to different structures related to the vessels, until the cedar oil is entirely replaced for greatest translucency. After treating in artificial oil of wintergreen (methyl salicylate), however, it is advisable to return the specimens to cedar-wood oil for prolonged keeping.

Instructive sections may be made by first transferring the specimens to oil of cloves, then through the following series for fifteen minutes each: Equal parts oil of cloves and thick celloidin; pure thick celloidin; harden in a block by chloroform bath; cut thick sections and mount on slide coated with mixture of clove oil and celloidin; fix with xylol. Thinner sections can be made after infiltrating the block with paraffin.

# MICRURGICAL TECHNIQUE FOR THE STUDY OF CELLULAR PHENOMENA

ROBERT CHAMBERS AND M. J. KOPAC

INTRODUCTION 62. APPARATUS AND METHODS 65. Micrurgical instruments 65. Choice of microscope and microscope accessories 70. Moist chamber and coverslips 74. Microneedles 78. Cellular microinjection apparatus and technique 86. Microinjection apparatus for renal corpuscles, blood capillaries, etc. 94. Dark-field illumination for micrurgical work 95. Microsaltbridges, microelectrodes and micromagnets 96. Additional accessories 97. APPLICATIONS OF THE MICRURGICAL TECHNIQUE 97. Protozoology and embryology 97. Cytology and histology 99. Tissue culture 101. Cellular physiology 101. Technique of sub-cooling cellular tissues 104. Isolation of microorganisms 105. Additional applications 106.

## I. Introduction

The micrurgical<sup>1</sup> or micromanipulative technique involves procedures which fall into two groups: (1) methods for operating with the free hand under low magnifications and (2) the more strictly, so-called, micrurgical methods which have been developed for the purpose of operating under the higher magnifications of the compound microscope. In this chapter only the latter methods will be considered.

The micromanipulator is an accurately constructed mechanism for the purpose of controlling the movements of minute needles and pipettes under the field of observation in a microscope. In the instruments constructed so far the three-dimensional movements imparted to the needles or pipettes are practically at right angles to one another.

The micromanipulator is either clamped to the stage of a microscope or is mounted on a pillar in front of or to one side of the microscope. The needles or pipettes are mounted in special holders on the instrument and project over the stage of the microscope into the field of the objectives. For comparatively low-power work the needles can be placed so that their tips project down toward the stage of the microscope between the objective and the object. This method has been used for inserting pipettes into renal corpuscles and tubules and into blood capillaries.

When high power objectives are used the operations must be performed with the tips of the needles or pipettes bent up and projecting into a hanging drop suspended from a coverslip which serves as the roof

<sup>1</sup> Micrurgy (*micro*s, small; *ergon*, work) is a term introduced by Péterfi (see footnote 10) to denote micromanipulative technique in the field of the microscope.

of the moist chamber (Fig. 8). Or if the inverted microscope (see Fig. 7) is available the tips of the needles or pipettes are bent down and project into a drop of fluid containing the material under investigation which is resting on the upper surface of a coverslip. The lack of any obstacle between the coverslip and the objective permits observation of the operations with the highest powers of the microscope. Sufficient illumination can be obtained by the use of special substage condensers having a free working distance commensurate with the height of the moist chamber.

The effect of introducing microneedles, micropipettes and microelectrodes into immediate contact with protoplasm has been studied by numerous investigators. To be sure, one cannot attempt to perform any of the operations known as microdissection without injuring the protoplasm to a certain extent. Because of this the micrurgical method has been severely criticised.<sup>2</sup> It is believed, however, that with careful micro-manipulation any injury that is created is still the slightest injury that can be inflicted experimentally on protoplasm. It does not seem necessary to abandon the micrurgical method because of the possibility of producing injuries. Brooks<sup>3</sup> has pointed out that "experimental biology consists in the production and study of abnormalities." It is to be remembered that these injuries must be reversible.

In all probability, injuries to protoplasm induced by micrurgical technique are more reversible than many of the other experimental effects frequently produced. Recovery from micrurgical experiments has been noted by many investigators. Gelfan<sup>4</sup> reports that streaming in *Nitella* is resumed shortly after the insertion of a micropipette. Indeed if the insertion is made carefully not only manipulation with a needle point but injections may be performed without the cessation of streaming. Taylor and Farber<sup>5</sup> were able to remove the micronucleus from *Euplotes* without destroying the organism. The only effect noted was the failure of the organism to reproduce. Chambers<sup>6</sup> noted that the region in a starfish egg even when injured reversibly by a microneedle becomes acid (pH 5.4), but that a return to the normal pH of 6.8 is made almost instantly. The injection of Na and K salts into the cytoplasm of amebas according to Chambers and Reznikoff<sup>7</sup> does not lead to any irreversible changes. Other instances may be given. The micrurgical method is still

<sup>2</sup> Heilbrunn, L. V. *Protoplasma Monographien*. Berlin, 1928, vol. 1.

<sup>3</sup> Brooks, S. C. *Protoplasma*, 8: 329, 1929.

<sup>4</sup> Gelfan, S. *Protoplasma*, 4: 192, 1928.

<sup>5</sup> Taylor, C. V., and Farber, W. P. *Univ. Calif. Publ. Zool.*, 26: 131, 1924.

<sup>6</sup> Chambers, R. In: Alexander: *Colloid Chemistry Theoretical and Applied*. N. Y., 1928, 2: 467.

<sup>7</sup> Chambers, R., and Reznikoff, P. *J. Gen. Physiol.*, 8: 369, 1926.



useful as long as the injury is recognized and the results are properly evaluated.

Undoubtedly thixotropic changes in the consistency of the proto-

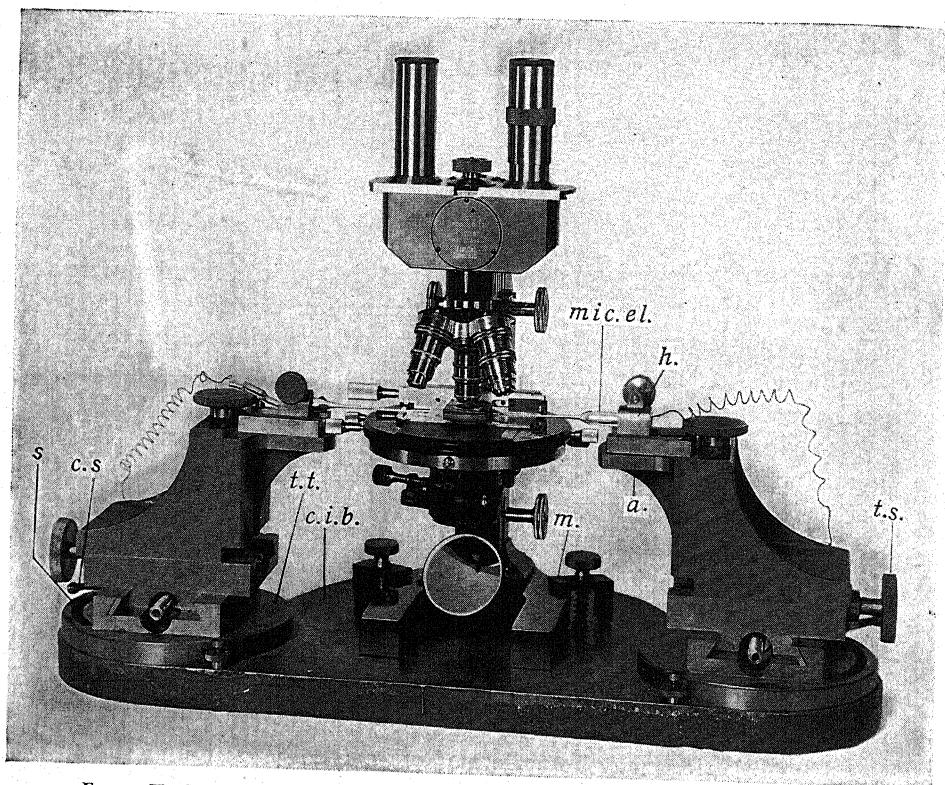


FIG. 1. Taylor micromanipulator equipped with microelectrodes. Moist chamber on stage of microscope is open at two ends for entry of microneedles, one from each side. *a*, Arm supporting holder for operative instrument; *c.i.b.*, cast iron base; *cs.*, clamp-screw for turntable; *h.*, holder for operative instrument; *m.*, clamp for microscope; *mic. el.*, microelectrode; *s*, slot in turntable; *t.s.*, thumbscrew of micromanipulator; *t.t.*, turntable. (From *Univ. Calif. Publ. Zool.*, 1925.)

plasm are caused by the insertion of microneedles into a cell. The use of micrurgical technique in the study of protoplasmic consistency is discussed by Seifriz.<sup>8</sup> Plowe<sup>9</sup> emphasizes the importance of noting changes in Brownian movement, aggregation of granules, swelling and other factors. These properties may be conveniently studied by using darkfield illumination. Even though protoplasm may be injured by the application

<sup>8</sup> Seifriz, W. *Bull. Nat. Res. Council*, No. 69, 229, 1929.

<sup>9</sup> Plowe, J. Q. *Protoplasma*, 12: 196, 1931.

of various micro-instruments, it is believed that if the protoplasm is continually observed and such factors as streaming, swelling, Brownian movement and evidences of cytolysis are noted, useful knowledge con-

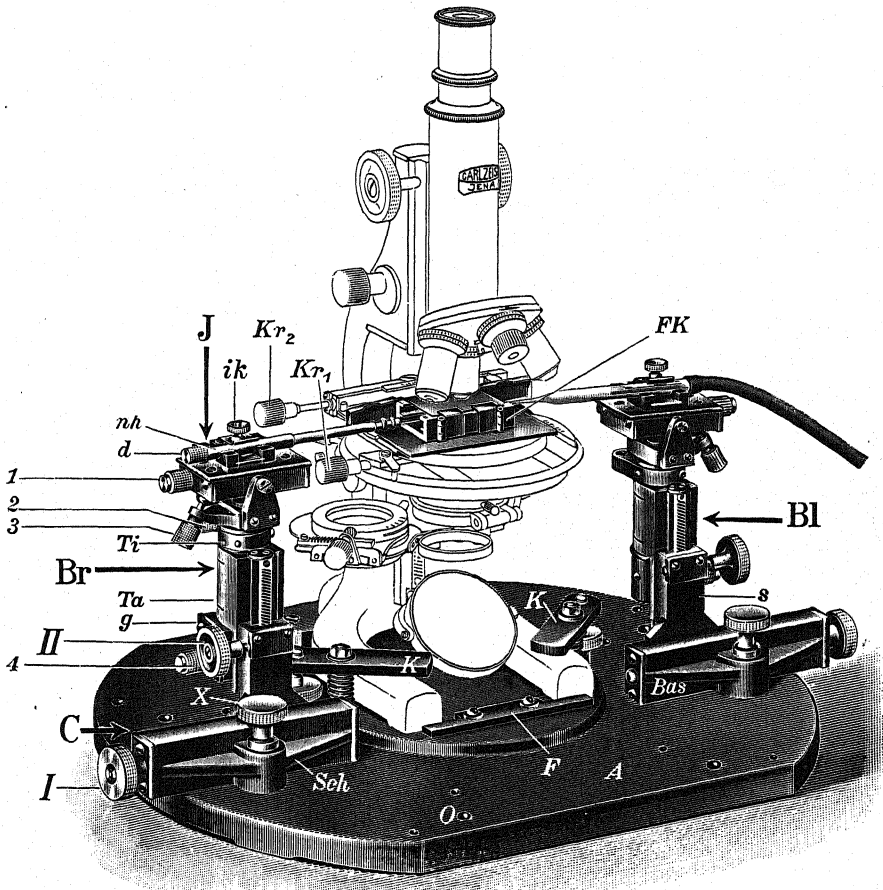


FIG. 2. Péterfi micromanipulator. Moist chamber is mounted similarly to Taylor's. One movement, B, is equipped with a microneedle, the other, also B, with a micro-pincette whose two tips can be separated or approximated by means of screws at base of pincette; A, common base for two or more movements and a microscope.

cerning cellular physiology and cellular structure may be obtained. It is important to recognize irreversible injuries. Whenever an injury becomes irreversible the value of the results must be discounted.

## II. Apparatus and Methods

1. **Micrurgical Instruments.** There are several forms of micro-manipulators in use at present, Taylor's, Péterfi's, Chambers', Fitz's and

Emerson's.<sup>10</sup> Taylor's instrument (Fig. 1), formerly manufactured by Mr. Val Arntzen of the University of California, is based on the same principle as that of the original Barber pipette holder.<sup>11</sup> The Taylor

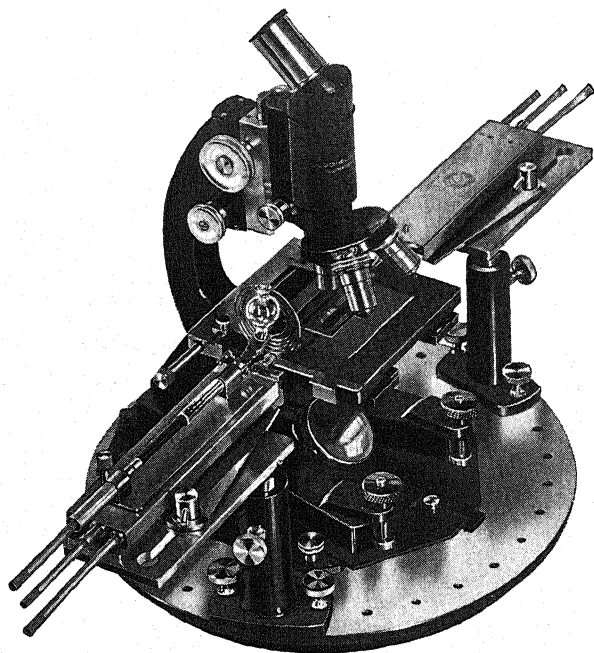


FIG. 3. Fitz micromanipulator. Two movements, right and left, and microscope mounted on a base; one movement equipped with microinjection syringe and special screw-feed mechanism for controlling syringe in cellular injections, etc.

micromanipulator, however, is far more stable and consists of two massive three-way movements mounted on a base which carries the microscope. It depends upon its mass and upon the precise hand-ground surface of contact for the accuracy of its movements which are produced by an arrangement of three metal slides perpendicular to each other and controlled by carefully cut and fitted feed screws. Péterfi's instrument (Fig. 2) (Zeiss) consists of either two, three or four pillars mounted on a common base. It is much lighter than Taylor's and de-

<sup>10</sup> Taylor, C. V. *Univ. Calif. Publ. Zool.*, 26: 443, 1925.

Péterfi, T. *Handb. microbiol. Technik*, 1923, p. 2471. Also in Abderhalden: *Handb. biol. Arbeitsmethoden*, Abt. v, 2: 479.

Chambers, R. *Anat. Rec.*, 24: 1, 1922. (Reprinted in *J. Roy. Micro. Soc.*, p. 373, 1922.)

Fitz, G. W. *Science*, 73: 72, 1931.

<sup>11</sup> Barber, M. A. *Philippine J. Science*, B, 9: 307, 1914. Rev. in *Ztschr. f. wiss. Mikr.*, 32: 82, 1915.

pendes for its movements on both sliding and rocking mechanisms. Fitz's instrument (Fig. 3) (Bausch and Lomb) is a recent development, in which the motions are produced by a cam or wedge action on semi-

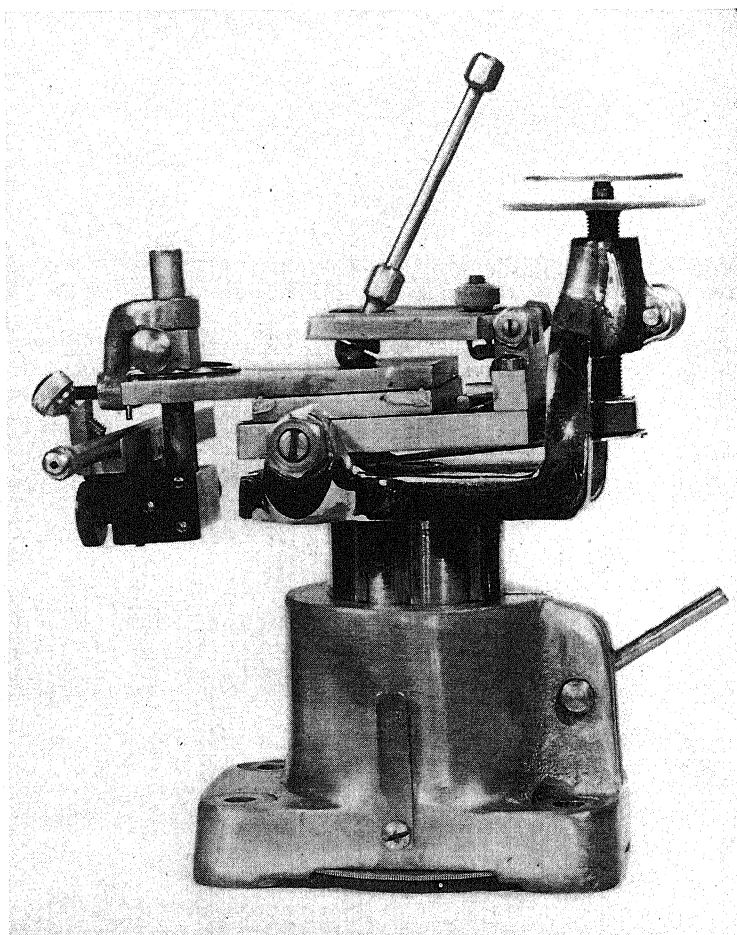


FIG. 4. Emerson micromanipulator. One micromanipulator movement showing lever which controls two horizontal motions, and coarse and fine feed-screw adjustments for vertical motion. Needle carrier is equipped with a rack and pinion for coarse adjustments. This unit together with other similar movements and a microscope may be mounted on a common base.

sliding components. Delicateness of movement is obtained by the use of differential feed screws. In the Emerson instrument (Fig. 4), manufactured by J. H. Emerson (Cambridge, Mass.), the movements are produced by manipulating one or two levers which in turn actuate the sliding components of the micromanipulator.

Chambers' instrument (Fig. 5) (Leitz) also consists of two or more pillars mounted on a common base. It is of about the same bulk as that of Péterfi's but depends for its fine movements upon the spreading apart

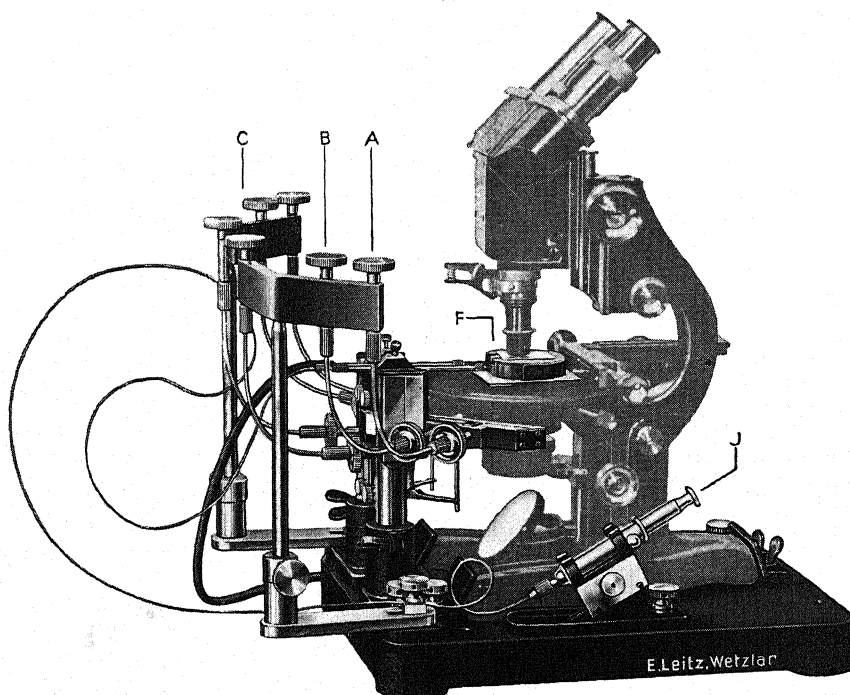


FIG. 5. Chambers micromanipulator. Two movements mounted in front of microscope on a common base; also showing remote control shafts for fine motions and microinjection apparatus with flexible tube and syringe, J, A and B, two horizontal motion feed-screw handles; C, vertical motion feed-screw handle; F, moist chamber open at front end for entry of microneedles.

of bars of rigid metal connected at their ends to form a z-like figure by resilient metal acting as spring hinges. The tips of the needles of Chambers' instrument move in arcs of large circles. Under the microscope the curvature of the arcs is not appreciable but this feature limits the position of the instrument in its spatial relation to the stage of the microscope. Further increase in the precision of the Chambers micromanipulator has been made possible by the introduction of remote fine operating controls attached to the three movements by flexible shafts. The use of these flexible shafts almost completely eliminates transmitting vibration to the three movements by the hand.

All the foregoing instruments have been used for most delicate opera-

tions. Theoretically, the one which uses the least amount of frictional surfaces in its fine movements should withstand the longest usage without impairment. In Chambers' instrument, due to its kinematic design, the frictional surfaces are at a minimum. The other instruments can be placed at any distance from the microscope stage without affecting the relative directions of the horizontal and vertical movements.<sup>12</sup>

It is an advantage but not an absolute necessity to have a rigid table upon which the instruments are to rest. In all instruments, the micromanipulator and the microscope are rigidly mounted on a common base so that the entire equipment may vibrate as a unit. However, if external vibrations are excessive, the hanging drop is frequently apt to vibrate out of phase with the microneedles. A mat of heavy felt or sponge rubber beneath the base will appreciably minimize this.

The movements of most instruments are adjustable so that they can be made to slide to a considerable distance from the microscope. This facilitates the frequent change of pipettes especially for bacteriological work. In Chambers' instrument (Fig. 6), this adjustment is a removable accessory which may be purchased with the instrument.<sup>13</sup> After the movement is brought back to its position close to the microscope it is rigidly clamped. This clamping feature is absent in Péterfi's instrument. In most cases the movements may be mounted either in front of or at the sides of the microscope. In the Chambers and Péterfi micromanipulators, four units may be mounted and used, thereby permitting the simultaneous control of four microneedles or micropipettes. Quite frequently a coarse movement is useful for placing minute hanging drops

<sup>12</sup> Other micromanipulators which have been described are those of:

Schmidt, H. D. *Amer. J. Med. Sc.*, n. s., 37: 2, 1859.

Chabry, H. J. *de l'anat. et phys.*, 23: 167, 1887.

Doty, H. A. J. *Applied Micro.*, 3: 991, 1900.

Schouten, S. L. *Ztsch. f. wiss. Mikr.*, 22: 10, 1905.

McClendon, J. F. *Biol. Bull.*, 12: 241, 1907.

Tchahotine, S. *Ztsch. f. wiss. Mikr.*, 29: 188, 1912.

Hecker, F. J. J. *Inf. Dis.*, 19: 306, 1916.

Malone, R. H. J. *Path. Bact.*, 22: 222, 1918.

Bishop, G., and Tharaldsen, C. *Amer. Nat.*, 55: 381, 1921.

Johnson, H. J. *Bact.*, 8: 573, 1923.

Briedigan, F. T., and Chang, T. M. J. *Lab. & Clin. Med.*, 9: 572, 1924.

Dunn, F. L. J. *Inf. Dis.*, 40: 383, 1927.

Florian, J. *Ztschr. f. wiss. Mikr.*, 45: 460, 1928.

Kopac, M. J. *Trans. Am. Micr. Soc.*, 47: 438, 1929.

McNeil, E., and Gullberg, J. E. *Science*, 74: 460, 1931.

de Fonbrune, P. C. R. *Acad. Sci.*, Séance, Oct. 10, 1932.

Reyniers, J. A. J. *Bact.*, 23: 183, 1932.

Buchthal, F., and Persson, C. J. *Scient. Instr.*, 13: 20, 1936.

<sup>13</sup> Wright, W. H., and McCoy, E. F. J. *Lab. & Clin. Med.*, 12: 3, 1927.

of fluid on the under surface of a coverslip. These accessories are at present made by two firms.<sup>14</sup>

2. Choice of Microscope and Microscope Accessories. The pil-

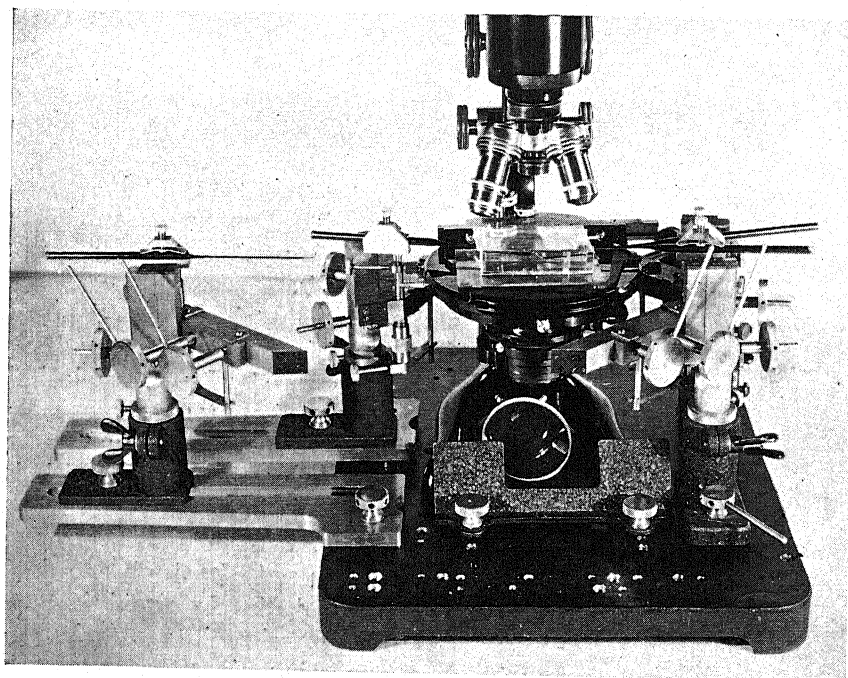


FIG. 6. Chambers' micromanipulator of four micro-movements. Two micro-movements on left side are equipped with sliding ways. One of these units is in position for micromanipulation and the other, on its sliding way, is in position for exchanging microneedle. Moist chamber, of glass, is open at two ends, permitting entry of four microneedles.

lars of the Chambers' instrument can be adjusted to the height of any standard microscope. Péterfi's, Fitz's and Emerson's micromanipulators are similarly adaptable but to a lesser degree. The Taylor micromanipulator is designed for use primarily with the large research model microscope stand.

A steady, well-controllable mechanical stage is essential in this technique because a great deal of the micro-operative work is done by moving the moist chamber with the mechanical stage. The latter should have a range which allows the moist chamber to be moved far enough so that the free edge of the coverslip roof of the chamber passes beyond the center of the microscopic field. This enables one to adjust fresh

<sup>14</sup> Leitz, rack and pinion micromanipulator. Zeiss, simplified micromanipulator.

needles and pipettes without the danger of striking them against the roof of the chamber. Mechanical stages with control heads on both right and left sides are extremely useful in micrurgical work.

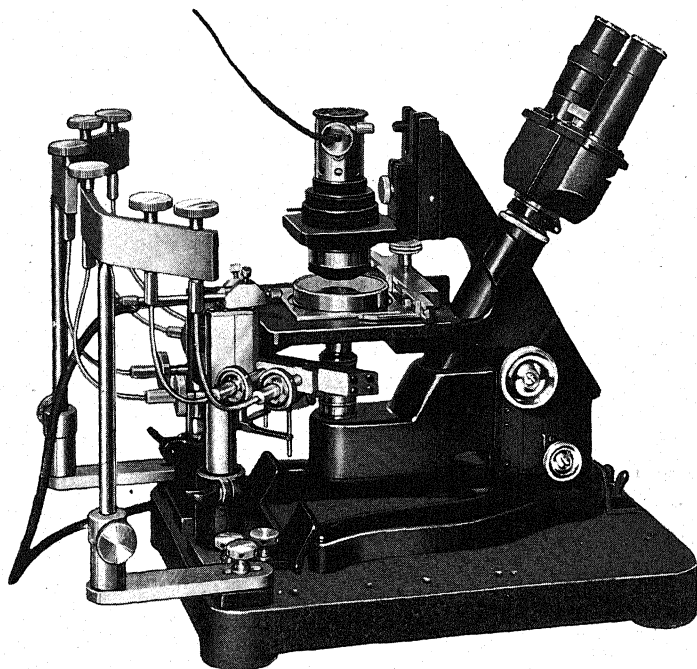


FIG. 7. Inverted microscope. Inverted microscope mounted on base with two micromanipulator movements. Note position of objective with clutch changer (may be replaced by revolving nosepiece) and illuminating condenser which has a built-in electric lamp.

Probably the best type of microscope stage is a circular rotary stage with a built-in mechanical stage. The rotary feature is frequently of considerable assistance for bringing the open end of the moist chamber into an advantageous position with respect to the shafts of the microneedles.

The binocular, mono-objective microscope is preferable to the monocular type. The equipment of oculars should include a good micrometer and a double demonstration ocular.

Recently Leitz has developed an inverted microscope for use with the micromanipulator (Fig. 7). The objective occupies a position under the coverslip, while the fluid drop containing the material under investigation lies upon it. The hanging-drop method provides considerable difficulty in obtaining large and flat spreading drops. Likewise many ob-



jects, particularly echinoderm ova, have a tendency to fall to the bottom of a large drop, thus making difficult the insertion of a microneedle or micropipette into them. When hanging drops are used they must be small and shallow to hold the material against the coverslip. This introduces the complication of undue evaporation. Small convex droplets act as lenses and the introduction of a microneedle into them causes considerable trouble optically. The creeping of the fluid around the microtip frequently tends to blot out the image in the microscope. With the inverted microscope a large amount of fluid may be used thereby offsetting rapid evaporation and eliminating the optical effects of tiny droplets. In fact a comparatively deep well of liquid may be used with a considerable length of the needle shaft completely immersed. In the inverted microscope the microneedles and micropipettes are manipulated above the coverslip and object. Above these is the superstage consisting of a condenser and a small illuminating lamp. Other types of illumination including darkfield may be used.

Since no obstacle between the coverslip and the objective exists, it is possible to use objectives of any type or power. Certain objectives are more useful than others. No optical complications arise from the use of lower power objectives.

The 16 mm. objective is always useful and the 32 mm. or a Leitz No. 2 objective is convenient for bringing the tips of the microneedles or pipettes into the microscopic field or for finding them when they are accidentally shifted out of the field. For larger objects, e.g., fresh water amebas, the Leitz No. 5 objective is a most useful one. It can be used with high power oculars and its free working distance is considerably greater than that of the 4 mm. objective. For still smaller objects, marine invertebrate ova for example, the Leitz No. 7a objective with an initial magnification of  $58\times$  is indispensable.

In attempting to use high power objectives several difficulties arise. The most important is the very restricted working distance. Another factor, very important theoretically, is the reduced numerical aperture of the illuminating condenser. This reduces the resolution very appreciably. There is no point in using objectives with a high numerical aperture. Because of the air space existing between the condenser and the object, the theoretical limiting numerical aperture is therefore 1.00. In practice a numerical aperture of more than 0.75 is not realized due to the extraordinary working distance of the condenser.

However, high power oil immersion objectives may be used with considerable profit particularly because of the increased illumination over that of high dry objectives. They are useful for work in which resolution of extremely minute bodies is not needed. A great advantage

of high power objectives is their restricted depth of focus. This property enables one to place the microtip vertically into a cell with far greater precision than is possible with lower power objectives possessing greater depths of focus. In micrurgical work the 3 mm. apochromatic oil immersion objective is to be recommended. The Zeiss HI 60 apochromatic water immersion objective is an excellent one provided due correction is always made for coverslip thickness. The free working distance of the objective is of prime importance.

In order to secure optimum illumination the focus of the substage condenser must reach the roof of the moist chamber which, for ordinary purposes, should not be over 10 mm. high. Leitz makes a brightfield condenser with a long working distance of 15 mm. and a numerical aperture of 0.75. A triple lens, or, better still, an achromatic, aplanatic condenser can be converted into a condenser having the required focal length by removing the top lens. In some makes the top lens has a screwing attachment for this purpose. The resulting numerical aperture will vary with different makes of condensers. In order to take advantage of the full focal length of the condenser the substage must be adjusted to allow the condenser to be brought flush with the upper surface of the microscope stage. A simple way to detect the focal point of a condenser is to blow some smoke on the upper surface of the brightly illuminated condenser whereupon the emerging cone of light is plainly seen. A better method is to place a block of uranium glass over the condenser. Lacking the uranium glass a thin-walled shallow dish filled with a dilute solution of eosin or fluorescein will serve.

A serviceable lighting system is a 100 watt electric bulb with a concentrated filament screened by a filter of Gage's daylight<sup>15</sup> (Corning's daylight) ground glass, and a spherical flask of water or a bull's-eye lens, placed between the light source and the mirror of the microscope. The position of the flask or bull's-eye can be determined by removing the ground glass filter and shifting the relative positions of the flask and of the lamp until a sharply defined image of the source of light can be thrown by the plane mirror of the microscope on a distant screen or wall.

The image can be seen either by placing a sheet of white paper on the face of the mirror or by projecting the image on the wall, by means of the mirror. The filter may be placed either between the light source and the flask or between the flask and the substage condenser. The brightest illumination is usually procured by the latter position.

For photomicrography and other uses, the Leitz "Monla" lamp gives excellent illumination. This is an incandescent illuminant using a low

<sup>15</sup> Gage, S. H., and Kingsbury, B. F. *Anat. Rec.*, 10: 527, 1915-16.

voltage, high amperage bulb. Since the filament is wound for 6 volts, the amount of wire is small, thus resulting in a very concentrated source of light. Practically, this is point source illumination.

The Ultropak illuminator<sup>16</sup> (Leitz) is a very useful accessory for micrurgical work.<sup>17</sup> This instrument is a vertical illuminator permitting the use of a large series of objectives. The light source, consisting of a low voltage Mignon lamp, is an integral part. Color, density or heat absorbing filters may be conveniently used. The entire unit is mounted on the body tube to replace the nosepiece. The substage need not be removed, in fact no changes are necessary except to remove the nosepiece. Each objective is equipped with a ring-type condenser. A slight rotation of this condenser changes the illumination from brightfield to darkfield without disturbing those adjustments previously made either with the microscope or with the micromanipulator. The Ultropak objectives, particularly the lower powers, work equally well with transmitted light as furnished by a substage condenser. This permits one to make the necessary preliminary adjustments of the microneedles using ordinary illumination. A most valuable feature of the Ultropak is the possibility of using high power oil immersion objectives and realizing their maximum numerical aperture of 1.00. The working distances of these high power objectives is ample for hanging-drop techniques.

Leitz makes an Ultropak micromanipulator, a simpler device for manipulating one microneedle or pipette, which is useful in the study of fibers and bacterial isolation work. This micromanipulator consists of a centering adaptor fitting into the condenser sleeve of the substage. Attached to the centering adaptor is the manipulator proper with a support for a needle or a pipette. Lateral movements are obtained by manipulating two screws functioning similarly to those used in centering a substage condenser. Vertical movement is obtained by manipulating the rack and pinion of the substage.

Finally, emphasis must be laid upon the fact that a study of living cellular phenomena, especially in experimental work in which the elaborated methods of fixing, staining and clearing of tissues have to be discarded, requires a thorough appreciation of the limits and possibilities of critical microscopic vision.<sup>18</sup>

**3. Moist Chamber and Coverslips.** There are two general types of moist chambers in use. One is open at both ends, the other at one end only. The former is used whenever the micromanipulator units are

<sup>16</sup> The Epi Condenser W, as made by Zeiss, is similar to the Ultropak.

<sup>17</sup> Kopac, M. J. *Science*, 82: 70, 1935.

<sup>18</sup> Coles, A. C. *Critical Microscopy*. Lond., 1921.

Beck, C., *The Microscope*, Lond., 1924.

mounted at the sides of the microscope, the latter with micromanipulator units mounted in front of the microscope.

The chamber designed by Péterfi (Zeiss) is open at both ends which

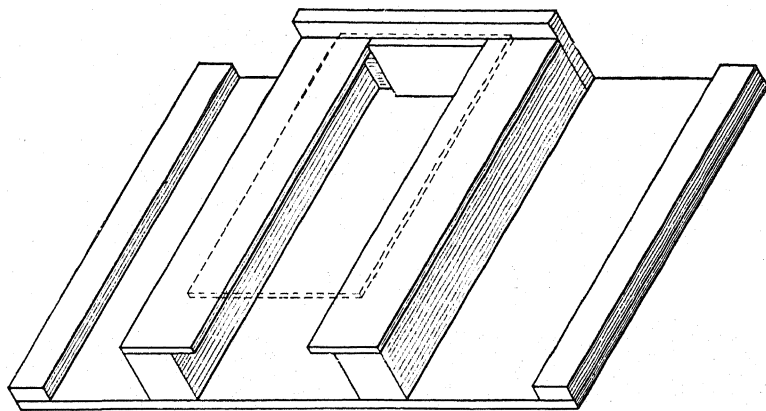


FIG. 8. Glass moist chamber open at one end.

can be closed with loose rubber sleeves. The microneedles are mounted so that one is on each side of the microscope and their tips are inserted into the chamber through closely fitting collars of the sleeves.

The type commonly used with Chambers' instrument is open at one end only and is shown in Figure 8. When this chamber is used the two micromanipulator units are placed so that both of the microneedles or pipettes pass into the chamber from the one end. The base of the chamber is a glass slide having a form which can be tightly gripped in the mechanical stage of the microscope. The walls are strips of glass or bakelite about 2 inches long, and of a height which is determined by the free working distance of the available condenser. In practice, this height is either 8 or 10 mm. One end of the chamber is closed with a strip of glass or bakelite of the same height as the side and backed by another strip 1 or 2 mm. higher. The trough of the chamber should be about 2 cm. wide. Strips of wet blotting or filter paper should be placed along the sides of the trough. Frequently it is preferable to have the entire floor of the moist chamber flooded with water, in which case a thin glass strip is cemented across the floor at the open end. A convenient size of chamber is one which can be roofed over by a 22 x 40 mm. coverslip. The slip is held in place with vaseline. Smaller coverslips may also be used, provided the rest of the chamber is covered with other strips of thin glass. It is often convenient to use two square coverslips, each 22 mm. wide.

When several droplets are to be placed on a single coverslip they may be kept separated by rings of wax. The convexity of the drop is diminished by the use of a ring. The rings can be readily stamped on the coverslip in the following way. A comparatively thin-walled glass or, preferably, a metal tube is selected, having an evenly surfaced end with a circumference of the size of the required rings. The end of the tube is heated momentarily in a flame and pressed first on a block of paraffin or beeswax and then on the coverslip. In this way slender rings of wax can be neatly stamped on the coverslip especially if the slip is kept cold by being laid on a glass plate over a wet towel. The coverslip must be carefully cleaned and dried in order to insure the adhesion of the wax to the glass.

Needham and Needham<sup>19</sup> use what they call a "flying coverslip." The chamber is completely covered with a long oblong coverslip to the underside of which is attached, by a film of water, a small coverslip with a hanging drop containing the tissue or cells to be operated upon. Taylor uses a glass or mica roof perforated with a hole of about 16 to 18 mm. in diameter. The material to be studied is mounted on a square or round coverslip which is then inverted over the hole and held in place with vaseline.

A circular hole is readily made in a glass slip or coverslip by wetting the glass with emery powder in water and boring the hole with a copper tube which has a circular knife edge on one end. This tube is mounted on a brace or a turning lathe. Thin sheets of mica or firm celluloid may be used and they can be perforated with great ease.

The chamber is purposely made rather long because of the evaporation which occurs at the open end. At a certain distance from the open end is a zone where the water condensation on the roof is at an optimum for maintaining the hanging drop which contains the cells or tissue. In order to prevent excessive condensation it is sometimes advisable to use saline solutions instead of water with which to moisten the chamber. The open end can be closed by means of a paraffined paper box with its sides cut out to accommodate the shafts of the microneedles. After being set in place the box is filled with vaseline thinned with oil.

A special type of hermetic chamber devised by Barber<sup>20</sup> has been developed by Needham and Needham<sup>21</sup> for dissecting and injecting cells in atmospheres of various gases. The chamber is closed at one end by a trough of mercury through which needles and pipettes on shafts with a u-bend pass into the chamber. The chamber is supplied with two inlets, one to flood the chamber with the gas and the other for a delivery pipette

<sup>19</sup> Needham, J., and Needham, D. M. *Proc. Roy. Soc.*, s. B, 98: 259, 1925.

<sup>20</sup> *Philippine J. Science*, B, 9: 307, 1914. Rev. in *Ztschr. f. wiss. Mikr.*, 32: 82, 1915.

<sup>21</sup> *Proc. Roy. Soc.*, B, 98: 259, 1925.

to deposit a hanging drop of a desired solution after the chamber has been filled with the gas. There is also an outlet for the air or gas to pass out. The chamber and shafts for the micropipettes and needles have been further elaborated with the aid of C. G. Grand of this laboratory and are described in a recent publication.<sup>22</sup>

Reyniers<sup>23</sup> has described a rather complicated moist chamber, entirely enclosed during work, in which a constant gaseous tension can be built up and maintained and which can be used with any micromanipulator. Pipettes may be changed without exposing the interior of the chamber. By using replaceable moist chambers any number of experiments may be carried out simultaneously with the same apparatus.

A desirable accessory is a moist chamber for preparing and teasing tissues before mounting for micromanipulation.<sup>24</sup> It consists of a small, bottomless box with a flat glass top and two sides of thin rubber. The box is removably mounted on a plate of glass on which it is held snugly by strips of glass cemented to the plate. A coverslip, with the tissue to be teased, is laid on the glass plate and the box placed over it. The entire chamber is then mounted on the stage of a dissecting microscope and the tissue is teased by means of dissecting needles thrust through the rubber sides of the box.

The cleaning of glass is a most important feature in this type of work in which everything depends not only upon cleanliness but also on the size and shape of the drops suspended from the coverslip and upon the condition of the glass rods and tubing from which the microneedles and pipettes are made.

Glass should be cleaned by boiling thoroughly in soapy water, rinsing and then immersing for hours in a cleaning mixture of saturated potassium dichromate (one part) and concentrated sulphuric acid (one part). It should then be washed thoroughly in running water, rinsed in ammoniated distilled water and kept in clean 95 per cent alcohol. When needed, the glass may be dried with heated air.

Another consideration to be guarded against is the possibility that the alkalinity of ordinary glass and the possible loss or accumulation of  $\text{CO}_2$  or  $\text{NH}_3$  in the hanging drop may seriously interfere with the chemical conditions in a droplet on a coverslip or in the micropipette. Soft glass tubing may be used provided the micropipette is rinsed in distilled water just before using. A flying quartz coverslip may be used to avoid the chemical effects of the glass. Pyrex glass, due to its rather high arsenic content and its excessive brittleness, is unsatisfactory.

<sup>22</sup> Cohen, B., Saunders, R., and Reznikoff, P. *J. Gen. Physiol.*, 11: 585, 1928.

<sup>23</sup> Reyniers, J. A. *Anat. Rec.*, 56: 295, 1933.

<sup>24</sup> Chambers, R. *Biol. Bull.*, 34: 121, 1918.

4. **Microneedles.** An automatic device for drawing microneedles and micropipettes (Fig. 9) described by DuBois<sup>25</sup> is now supplied by Leitz. It is based on a method developed by Keith Lucas<sup>26</sup> for making

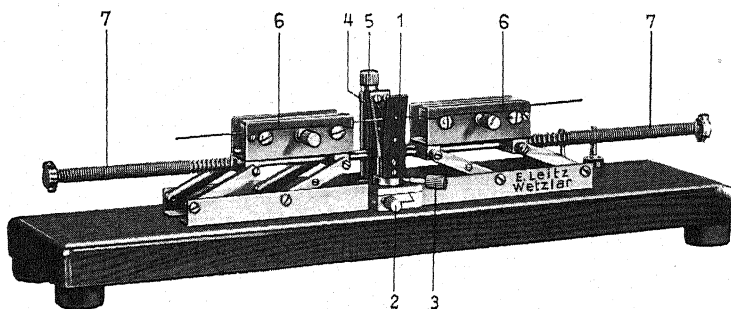


FIG. 9. DuBois needle pulling machine. Parts denoted by reference numbers: 1, platinum loop carrier; 2, 3, screws for adjusting position of loop carrier; 4, 5, mechanism for height adjustment; 6, clamp tension device for holding glass capillary; 7, tension spring.

fine glass capillaries. A piece of capillary tubing is mounted in the apparatus and clamped by a taut spring. A platinum loop surrounding a small portion of the capillary is heated electrically. As the glass begins to melt, it is lifted out of the heat and immediately drawn out resulting in two identical microtips.

An excellent needle making machine has been recently developed by Dr. L. G. Livingston (Biological Laboratories, Harvard University). A platinum loop is used for melting the glass capillaries. This apparatus is distinguished in two ways, first, by its extreme smoothness and precision of action, and second, in the possibility of making and duplicating needle and pipette points of almost any desired shape.

A simpler and effective mechanical device for making needles has been described by Reyniers.<sup>27</sup>

With some practice, however, it is possible to make microneedles and micropipettes by free hand. The equipment for this purpose is shown in Figure 10 and detailed description of the method follows.

*a. Making the Microneedles.* (1) *The Glass.* The needles are made from glass-rodlets made from soft or hard glass. Considerable time is saved by using stock precision drawn glass capillary rods. We use soft glass rodlets 0.85 mm. in external diameter for making microneedles. The technique for making needles of soft glass is readily mastered. It is

<sup>25</sup> DuBois, D. *Science*, 73: 344, 1931.

<sup>26</sup> Lucas, K. J. *Physiol.*, vol. 37: 1908, *Proc. of Soc.*, xxviii-xxx.

<sup>27</sup> Reyniers, J. A. J. *Bact.*, 26: 251, 1933.

somewhat more difficult to make them of hard glass but they tend to be more durable.

(2) If precision drawn capillaries are not available, the following

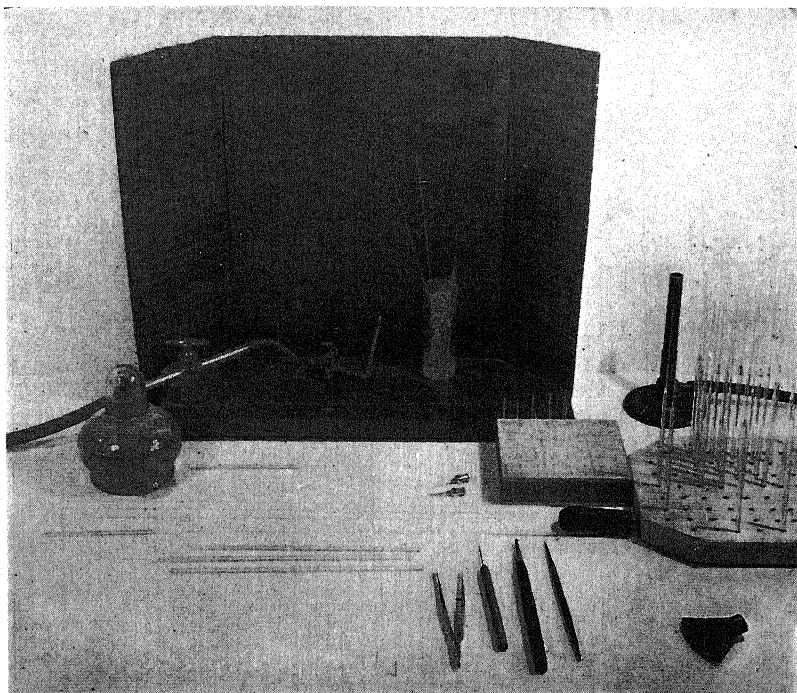


FIG. 10. Equipment for making microneedles and pipettes. In glass vial by gas microburner are several lengths of glass capillaries from which needles and pipettes are to be made. In foreground are instruments (flat-tipped forceps and steel, dissecting needle), pieces of glass tubing and rods (3-4 mm. in diameter), some of which have been drawn into capillaries. To right are two wooden stands with holes. One supports a supply of glass rods with capillaries, ends of which have been drawn into microneedles. On smaller stand is a supply of micropipettes. Ordinary soft and hard glass capillaries are drawn in a Bunsen gas flame. Quartz capillaries have to be drawn in an oxy-acetylene flame.

method may be used. Glass rods or tubes 3 to 5 mm. in diameter may be drawn to capillary dimensions in the flame or a Bunsen burner. The capillary size should be held to an external diameter ranging between 0.5 and 1.0 mm. Fine microtips cannot be drawn out of capillaries larger than 1 mm., while capillaries finer than 0.5 mm. tend to make the shaft of the microneedle too fragile. If precision needle holders (described later) are used, the ideal capillary size is 0.8 to 0.85 mm. in diameter.

(3) The microburner (Fig. 10) is made from a piece of hard glass



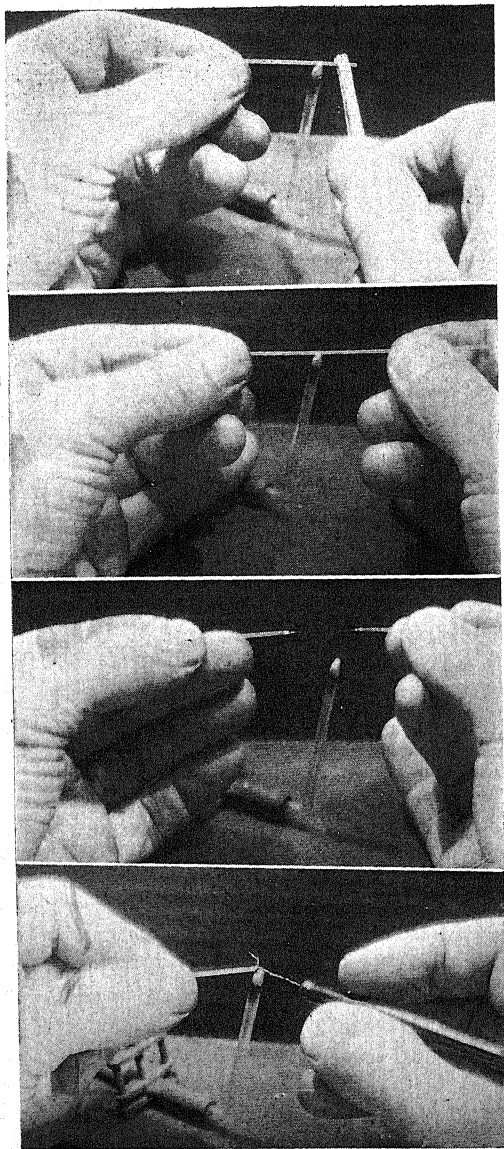


FIG. 11. Four photographs showing various stages in making microneedles and pipettes from a glass capillary. A, short capillary being held by forceps over microburner preparatory to drawing out a needle point. B, similar procedure with a long capillary held by both hands. C, tug which separates heated capillary in formation of needle tips. D, bending shaft of microneedle or pipette in microflame.

tubing bent almost at a right angle and with the upturned end closed except for almost the smallest possible aperture that will retain a flame. This can be done by heat-softening one end of the tube and pinching it with a pair of forceps or by allowing the end to melt down to a fine aperture. When set in place the tip of the burner should stand at a height somewhat less than the width of the operator's palm measured across the base of his fingers, (cf. Fig. 11). The size of the flame is regulated by one or two Hoffman screw clamps on the rubber tube connecting the burner with the gas jet. A steel hypodermic needle mounted in a rubber tube will also serve as a microburner.

#### (4) The Microtips.

(a) The Flame. Lower the flame of the microburner to almost the smallest flame that will remain lighted. The microburner should be protected from draughts of air and kept in semi-darkness or against a mahogany red or black background so that the flame will show up to best advantage.

(b) Length of Shaft. The shaft, when mounted, should be about 5 to 6 cm. long. This length depends upon the length of the moist chamber and the distance of the field of the microscope from the needle holder of the micro-manipulator. If the capillary shaft is too short its holder will extend into the moist chamber and so impede the range of movement. In practice, it is well to have the shaft 8 to 10 cm. long. The excess length may be trimmed just before mounting into the needle holder by scratching the capillary with a diamond point or a carborundum crystal and breaking at that place. A clean break will aid in preventing possible vertical displacement of the microtip (see p. 89).

(c) Drawing the Microtip. To make the microtip, hold the capillary tube or rodlet in the left hand and grasp the other end either with forceps or with the thumb and forefinger of the right hand. No special precaution is required regarding the length as the shaft is later cut to the proper size.

While pulling gently and steadily with the right hand bring the capillary just over the microflame, (Fig. 11 A, B).

As the glass is softened by the heat, lift it slowly from the flame while pulling slightly more than at first, but not too strongly. The hands should rest on the table during the procedure and the pulling and lifting done by turning them slightly outward (Fig 11, c). The capillary will separate with a slight tug—a feeling much like that experienced when a taut thread, held in the fingers, is parted in a small flame. If the needle is properly made, as in Figure 12, c, d, it will taper to a fine rigid tip. Everything depends upon the amount of heat used and the timing of the added pull, and these vary slightly with the height of the flame and the diameter of the capillary. With a little experience, one can usually tell when a proper tip is made by the peculiar feeling just described. If too little heat is used and the pull made too suddenly, the capillary may part with a snap and the tip will be found broken off short or if the capillary is hollow, rounded off bluntly with a short protruding hair. If too much heat is

used, the capillary will draw out into a long flexible, unserviceable hair (Fig. 12, *e*). The type of needle tip and its serviceability are determined upon examination through a low power of the microscope.

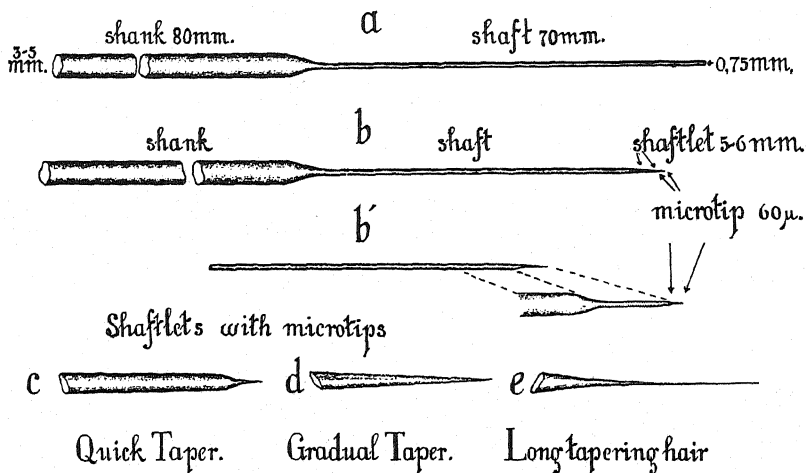


FIG. 12. Making of microneedles. *a*, glass shank (3-5 mm. in diameter) drawn out into a capillary about 0.75 mm. in diameter and 70 mm. long; *b* and *b'*, capillary shaft with and without shank. End of shaft is drawn out, in a microflame, into a shaftlet, 5-6 mm. long, and a microtip about 60 $\mu$  long; *c*, shaftlet tapered quickly into an elongated microtip (quick taper); *d*, shaftlet with a gradually tapering microtip (gradual taper); *e*, unserviceable, long, tapering hair.

(*d*) Types of Microtips. A successfully made microneedle (Fig. 12, *b*) on the end of a capillary shaft consists of a shaftlet and a microtip. The needles are of various types according to the form and size of the shaftlet and of the tip. It is usually impossible to tell beforehand which of these types will be made when drawing them out over the microflame.

The serviceable needles are classified into two general types, one, the quick taper (Fig. 12, *c*) in which the diameter of the shaftlet decreases more or less rapidly and then extends as a slender, elongated tip, and the other, the gradual taper (Fig. 12, *d*) in which the shaftlet tapers gradually to the tip. Both types may be further classified as coarse, medium and slender. Some actual measurements from typical needles are given in Table I.

With experience the operator will soon learn to appreciate the type of needles best adapted to his special requirements.

*b. Bending the Shaft.* After a suitable microtip is made the needle is bent at an angle over the microflame (Fig. 11, *b*). The bend should be anywhere between a right and an obtuse angle of about 135° or more. In Figure 13 several different types of bends are shown. A rigid needle

results if the bend is on the shaft just below the slender shaftlet (Fig. 13, *a*). This type of needle, especially if the bend approaches a right angle, is in danger of being easily broken when the tip meets the cover-

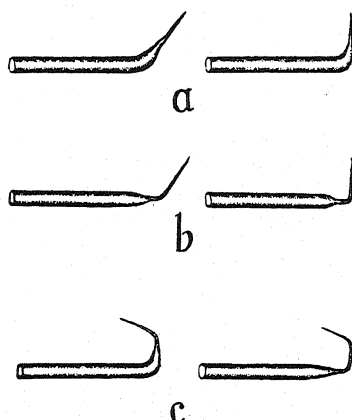


FIG. 13. Bending of shafts of microneedles. *a*, Rigid needles with bend on shaft; *b*, yielding needles with bend on shaftlet; *c*, rigid and yielding needles with shaftlets bent on themselves (reflex type).

slip. A more resilient and yielding type results when the bend is made on the shaftlet (Fig. 13, *b*) especially when the angle of the bend is obtuse. A type of needle (Fig. 13, *c*) in which the shaftlet is bent upon itself, is well adapted for cutting or moving a cell about.

The method of bending varies with the difference in the length of the shaftlet. If the shaftlet is long enough to be included in the knee of the bend, the heat of the microflame is often sufficient in itself to produce the bend. A metal needle or a broad-tipped forceps serves not only to protect the tip from the flame during the bending process but also to conduct away the heat which may otherwise completely melt the shaftlet. If the shaftlet is short the knee of the bend is made on the capillary shaft beyond the shaftlet and this requires pushing with the steel needle or with the forceps. The height of the portion of the needle beyond the bend should not be over 4 or 5 mm. for use in a moist chamber 10 mm. high.

The grasping surfaces of the forceps used in drawing out the needles should be more or less parallel. In order that the surfaces may grip without breaking the glass it is advisable to coat them with a thin layer of Canada balsam or varnish.

Fine points of shaftlets of serviceable length are not made from capillaries of a diameter much greater than 0.85 mm. Therefore, if a comparatively stiff shaft is required start with a thick capillary and draw out the

tip into a thinner capillary. This thinner capillary may then be used for making the microneedle.

*c. Mounting the Needles.* A superior arrangement is to use the pre-

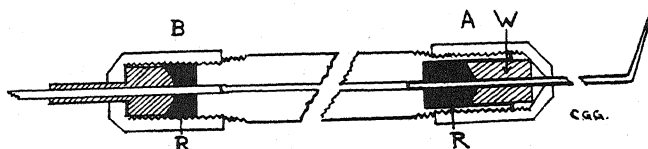


FIG. 14. Schematic figure (twice actual size) showing construction of precision needle holder. A and B, metal screw caps. W, a brass washer, concave on one end, which is forced against rubber packing, R, by screw cap, A. Micropipette is mounted on A end, while flexible metal tube leading to a syringe, with its rubber packing R, is mounted on B end of holder.

cision needle holder (Fig. 14) (Leitz) as designed by Dr. Richard Frank formerly of this laboratory. This device is a metal tube about 3 inches long and about 4 mm. in diameter fitted with rubber washers and screw caps at both ends. One end carries the microneedle or micropipette while the other end is free. When used in microinjection work the free end is fitted to a flexible metal tube leading to a syringe (described later). The shaft of the needle or pipette is inserted through the rubber washer in the nut and the nut is tightened by turning with a thumb and forefinger. This arrangement firmly clamps the needle or pipette in place and is also leak proof to the fluid used in the microinjection system (see p. 87). With the needle mounted in the precision holder, the holder is then clamped to the carrier on the micromanipulator. The needle is now ready to be adjusted within the microscopic field.

First one needle is coarsely adjusted by bringing it into the cone of light emerging from the substage condenser. Secondly, the tip of the needle is centered, first with the Leitz No. 2 objective (usually by hand) and afterwards with the 16 mm. (using the micromanipulator movements). A coverslip with a hanging drop of water is placed drop down on the moist chamber. This should be done outside the microscopic field and beyond the needle point. While the tip of the needle is being kept under observation through the 16 mm. objective the moist chamber is moved with the mechanical stage, until the edge of the coverslip appears into view. The tip of the needle should now be raised or lowered with the coarse vertical adjustments of the micromanipulator<sup>28</sup> to a level so that the cover glass may be moved over the tip without touching it. The hanging drop is now brought into the microscopic field, and the microtip is

<sup>28</sup> This procedure applies particularly to the Chambers micromanipulator.

further adjusted by using the coarse movement so that the tip almost but not quite touches the bottom of the hanging drop. Now by using the fine vertical movement, the microtip can be brought into the hanging drop.

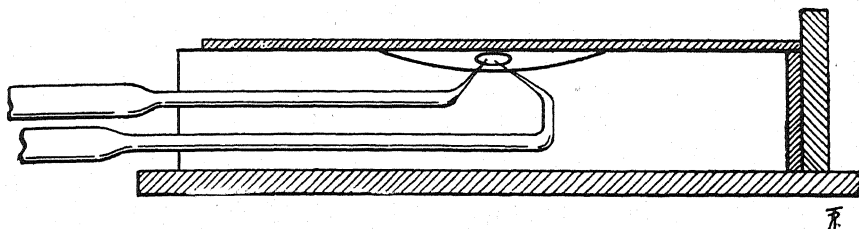


FIG. 15. Needles mounted in a moist chamber. Side view of moist chamber to show two microneedles with their tips in a hanging drop suspended from a coverslip which roofs the moist chamber.

On the Chambers micromanipulator the microtip should completely clear the bottom of the hanging drop when the fine vertical movement is brought to rest. The details of this last procedure will vary somewhat with the type of instrument and moist chamber available.

When once the first needle is properly adjusted with respect to elevation it is a simple matter to adjust the second needle point to the same elevation. Here the first needle is used as a guide. In microinjection the needle is frequently used only for the purpose of adjusting the micropipette to the correct elevation. A good needle, when once proved to be resilient and strong, can be used repeatedly.

When two microneedles are to be used, one may be bent backward and the other forward (Fig. 15). In using a micropipette in conjunction with a microneedle, it is advisable to have the microneedle bent backward (reflex type) and the micropipette bent forward (one bend). The tip of the reflex microneedle should not lie directly over the shaft of the capillary so that the latter will not be in the way of the light. For a cutting needle the angle at which the shaftlet is bent back on itself can be ascertained only by experience, for, if it is too near the horizontal, the tip, as it is raised against the coverslip, will tend to bend down instead of cutting up into the cell to be operated on. If the angle is too far from the horizontal, the cell will slip away from the needle.

*d. Method for Placing Microtip in Field of High Power Objectives.* More often than not, the objectives on a revolving nosepiece are neither centered nor parfocal. Thus if a microtip is centered in the field of a low power objective, it may be out of the field of a high power objective and its focal plane may be difficult to find. The following method is simple and effective. Mount a jagged edged coverslip on the moist chamber and

search for an easily identifiable portion of the edge in the field of the highest power objective which is to be used. Place this portion short of the center of the field. Now with the low power (Leitz No. 2 objective or its equivalent) place the tip of the needle as close as possible to the selected portion of the coverslip and adjust its level for correct elevation. On replacing the low for the high power lens, the tip of the needle will be in the field. When using the double instrument, one microneedle should be adjusted for position and elevation and kept there to serve as a guide for placing the tip of the other needle or micropipette.

e. *Other Methods for Obtaining Needle Tips.* Another good method for making the needle points is that of Chabry.<sup>29</sup> The tip of a glass capillary is brought into contact with a heated mass of glass (or any incandescent body to which glass will adhere) and suddenly drawn away. For an incandescent body Chabry used the blade of a platinum knife. The capillary is held in a groove on a stand a few centimeters from the platinum blade. This blade is then heated to a dull glow, and the capillary slid in the groove until the tip touches the glowing metal when it is instantly slid back. The sliding of the capillary in a stationary groove insures a straight taper.

Péterfi<sup>30</sup> has an ingenious microcautery device (incandescent platinum filament) for drawing glass points and making micro-hooks out of microneedles in the field of a microscope.

Needle points may also be made by grinding the end of a fine wire. To produce chemical effects in a cell, McClendon<sup>31</sup> used a copper wire ground to a point and further sharpened by erosion in acid. The chemical injury produced by metal needles limit their use for the dissection of living cells.

Péterfi described the use of scales of butterfly wings cemented to the tips of microneedles, et cetera. A useful cement for this work is made by dissolving celluloid in amyl acetate. Chabry also suggested the use of insect mouthparts, annelidan bristles and the spicules of sponges for the tips of microdissection needles. Barber<sup>32</sup> suggested fine pointed needle crystals, and the sharp, stiff hairs on the body of a house fly. A pipette with a fairly large opening is made and the hair or crystal is drawn partly into it. The fine point projecting from the tip of the pipette is then used as a probe or a dissecting needle.

##### 5. Cellular Microinjection Apparatus and Technique. The micro-

<sup>29</sup> Chabry, L. *J. d'anat. physiol.*, 23: 167, 1887.

<sup>30</sup> Péterfi, T. *Handb. microbiol. Technik*, p. 2471, 1923. *Hand. biol. Arbeitsmethoden (Abderhalden)*, Abt. v, 2: 479.

<sup>31</sup> McClendon, J. F. *J. Exp. Zool.*, 6: 265, 1909.

<sup>32</sup> Philippine *J. Science*, B, 9: 307, 1914. Rev. in *Ztschr. f. wiss. Mikr.*, 32: 82, 1915.

injection apparatus is used with a micromanipulator for the purpose of injecting aqueous or non-aqueous fluids and suspensions into the cytoplasm, vacuoles or nuclei of living cells. The diameter of the bore of the micropipettes may be anywhere from less than half a micron to more, according to the required type of microinjection.

The ability to microinject depends not only on the fineness of the micropipettes but also on the accurate control of pressure and the surface tension conditions of the fluids to be injected.

In the methods described by Barber and by Péterfi<sup>33</sup> the driving force depends upon the effect of heat and cold on the expansion of mercury on the one hand and air on the other. For finer work it is necessary to have a more dependable control over the pressure than is afforded by these means.

More serviceable methods are described below:

*a. Chambers Injection Apparatus.* This apparatus (Fig. 5) consists of the following parts: (1) a glass Luer-type syringe (1 to 2 ml. capacity) preferably with a metal nozzle; (2) a capillary tube of flexible metal (less than 2 mm. outside diameter and about 1 meter long); (3) a metal adaptor to join the syringe to the flexible tube (usually a hypodermic needle with the needle portion removed); (4) a precision micropipette holder (see p. 84) which joins the flexible tube to the micropipette; and (5) a syringe socket or holder for fastening the syringe to the base of the micromanipulator. The plunger of the syringe is controlled generally by hand. In certain cases a screw or lever feed may be used to an advantage.

The apparatus is assembled as follows:

The syringe and the lower end of the flexible brass tube, which connects with it, are clamped in place close to the base of the microscope. The flexible tube, running from the base clamp (Fig. 5) is curved and bent into a large loop until the precision needle holder at its upper end lies with no constraint in the carrier of the micromanipulator where it is to be clamped. To prevent leakage it is advisable to apply a thin film of Lubriseal on the nozzle of the syringe. Unless supplied clean the entire unit should be thoroughly flushed with xylol and rinsed with alcohol to remove any grease that might be present, especially in the tube. The syringe, the flexible tube and the precision needle holder are then completely filled with water (boiled or distilled) and the plunger pushed at least half way into the syringe. The entire system is thus full of water except for the micropipette which is to be set into the precision needle holder. Lastly a selected micropipette is mounted in the precision needle holder and the system is tested for leaks by compressing the plunger on the syringe.

<sup>33</sup> See footnotes 30 and 32.



Needham and Needham<sup>34</sup> have introduced a useful modification whereby the syringe can be filled by a continuous supply of water. Between the syringe and the flexible tube there are inserted two three-way stopcocks, one leading to a reservoir of distilled water, and the other opening to the exterior. By opening the former the syringe can be filled by pulling out the plunger; by opening the latter the liquid in the syringe can be expelled.

(1) Micropipettes. The micropipettes are made from hollow, thin-walled glass capillaries drawn to needle points as described on pp. 78 to 86 (cf. Fig. 13). An essential precaution in the bending of the shafts of micropipettes is to maintain a wide lumen at the bend. The bend should always be inspected with a low power microscope to determine whether the lumen is wide open at this point. The conversion into micropipettes is done, just before using, by breaking off their tips (see p. 89).

Hard or alkali-free glass capillaries are used for two reasons: (1) the brittleness of the hard glass enables one to break off the tip with greater precision, and (2) hard glass can be more readily cleaned and rendered alkali-free. Soft or soda-glass tubing may be used if care is taken to wash the pipette in clean distilled water just before using. Serviceable capillaries are drawn with an external diameter of 0.8 mm. and may be drawn by hand or supplied by a glass blower. Prior to drawing the glass should be carefully cleaned. The ends of the capillaries should be sealed to keep clean. Jena (one red stripe) glass has excellent qualities for micropipettes. The use of thin-walled capillaries is to insure having the largest bore possible in the slender shaftlet of the pipette. Sometimes it is more convenient to have a pipette with stouter walls. Such pipettes are less readily broken, but owing to the smaller sized lumen, run the risk of quickly clogging. The best pipette for general use is of the quick tapering type (Fig. 12, c, and Table 1). Its aperture, when first made, may be less than half a micron in diameter. Fluids will flow through it with ease provided the tip is dipping into a fluid of similar nature. The comparatively broad shaftlet close to the tip serves to supply pressure which enables one frequently to force open a clogged microtip.

(2) Mounting the Micropipettes. The former method of mounting micropipettes was to seal them with soft deKhotinsky's cement<sup>35</sup> to the base of a hypodermic needle which could be accommodated by a metal shank attached to the flexible tube. This method is inconvenient. Instead we use the precision needle holder described on page 84. It is possible to remove a broken pipette and insert a fresh one in less than a minute's time.

<sup>34</sup> *Proc. Roy. Soc., S. B.*, 98: 259, 1925.

<sup>35</sup> A special type of resinous cement used commonly in the laboratory.

Just before inserting the pipette into the needle holder a droplet of water should be expelled. Then the open end of the pipette shaft should be touched to this droplet so that some water will enter the capillary. Care should be taken not to insert the open end of the capillary too far into the needle holder. In most cases the lumen of the rubber washer is not concentric and if the shaft of the pipette projects beyond the rubber washer the microtip will undergo appreciable vertical displacement when pressure is applied from the syringe. By having the end of the pipette shaft flush with the end of the rubber washer this vertical displacement is less likely to occur.

(3) Opening the Microtip. After the micropipette has been mounted in the needle holder and the injection system tested for leaks, the microtip is adjusted in the microscopic field and also vertically with respect to the level of the coverslip. A microneedle previously adjusted for elevation is used as the guide for placing the micropipette. If the column of water, forced into the shaft of the micropipette by compressing the syringe, does not return to its original level upon releasing the pressure, the micropipette should be replaced for the tip of the pipette must have broken and is in all probability too large.

The intact microtip is now brought into the hanging drop of the fluid to be used for injection, and is converted into an open micropipette by raising the tip very cautiously and breaking it against the lower surface of the coverslip. Three precautionary measures are necessary during this procedure: first, the tip should be broken while under observation through the objective which is to be used for the injection. This insures against breaking off too much of the tip. The second precaution is to use the tip of the microneedle, mounted in the accompanying micromanipulator movement, to orient oneself regarding the proximity of the under surface of the coverslip. In other words, the needle should serve as an advance guard of the pipette to minimize the chance of damaging the latter. Finally, it is advisable to keep the plunger of the syringe under pressure while the tip of the pipette is being broken. This lessens the risk of clogging the pipette at the very start.

The injection fluid is now drawn into the pipette by exerting a gentle pull on the plunger of the syringe. Only a small amount should be drawn in. Rarely should the fluid pass the bend of the micropipette.

Operating the plunger of the syringe for expelling precise quantities of fluid from the micropipette requires considerable practice. An essential precaution for controlling the delivery of minute quantities is to have a microtip of the rapidly tapering type (p. 82, Fig. 12, c). Movement of the plunger of the syringe should be performed by gently rotating the plunger between the thumb and forefinger with the wrist firmly steadied

on the table. In practice, the syringe is mounted on the left side of the microscope, while the micropipette is mounted on the right micromanipulator movement. The location of the syringe with respect to the right or left side of the microscope is arbitrary, but the micropipette should always be mounted on the micromanipulator movement opposite to the side on which the syringe is mounted. Thus one can operate the micromanipulator movement holding the micropipette and the syringe simultaneously with the two hands.

From the preceding description it is evident that the injection fluid in the tip of the micropipette is separated from the water in the injection tube by a column of air in the shaft of the pipette. This point is further discussed later.

The hanging drop should be as free as possible from suspended particles which may clog the pipette. Only carefully filtered solutions should be used. Particles, especially those of dust, tend to sink and will in time accumulate in the lower surface of the deepest region of the drop. Therefore, especially with a very fine pipette, always fill the pipette while its tip is far up in the drop, and when removing it from the drop, be sure not to lower it through the deepest region where the particles tend to accumulate.

*b. Taylor's Injection Apparatus.*<sup>36</sup> This apparatus (Fig. 16) was designed to be mounted directly on one of the movements of the Taylor micromanipulator. Injection is performed by applying pressure on a column of mercury by means of a feed screw pressing against a thick rubber plug. The glass, h-shaped tube is filled with mercury after being first evacuated.

The micropipette is cemented to the glass shank with deKhotinsky's cement. Care must be taken to avoid boiling the cement while sealing the micropipette to the shank. If the gas from the cement escapes into the mercury there is a strong possibility of a break occurring in the mercury column within the micropipette shaft. After the micropipette is cemented in place, mercury is forced to the tip of the pipette by turning the coarse feed screw. Microinjection into a cell is performed by turning the fine feed screw. Fluids are drawn into the micropipette by reversing the feed screws.

*c. McNeil-Gullberg*<sup>37</sup> *Micropipette.* This instrument is a modification of the Taylor microinjection apparatus. It is smaller and makes use of a lever and thereby removes the screw from the direct line of the pipette. A more delicate control of the pressure is obtained and the movements of the hand are not transmitted to the micropipette to any

<sup>36</sup> *Univ. Calif. Publ. Zool.*, 26: 443, 1925.

<sup>37</sup> *Science*, 74: 460, 1931.

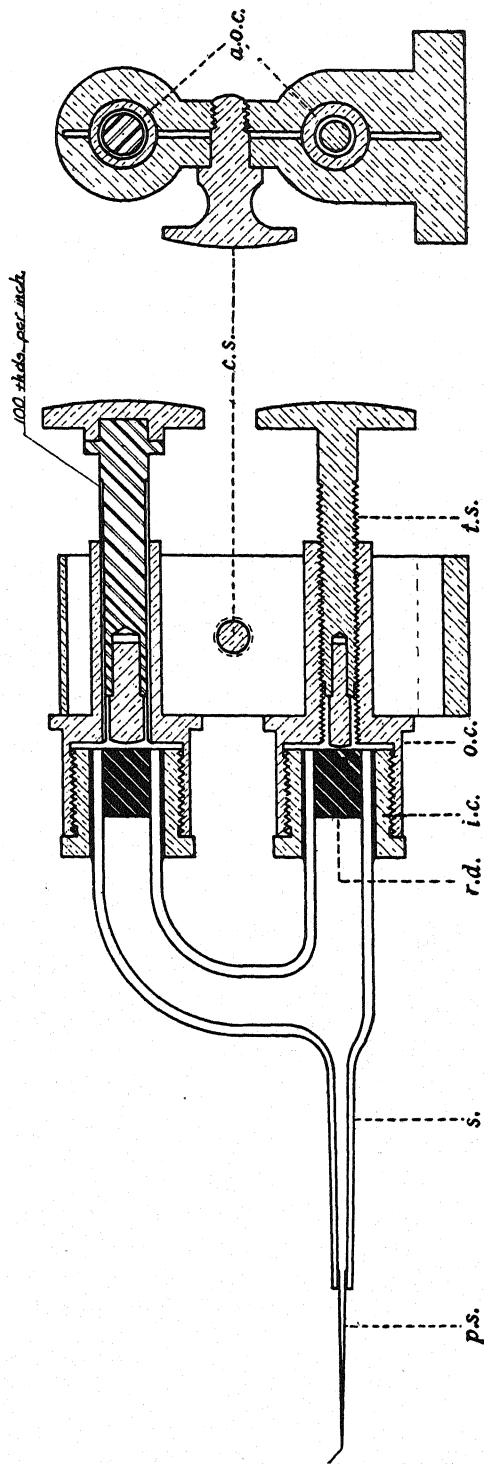


FIG. 16. Taylor's microinjection apparatus furnished with coarse and fine screws; *c.s.*, clamp screw; *i.c.*, metal inner cap; *o.c.*, metal outer cap; *p.s.*, micropipette shaft; *s.*, glass shank into which micropipette shaft is inserted and cemented, and which is later filled with mercury; *r.d.*, rubber diaphragm; *t.s.*, thumb screw. Massive construction of Taylor's micromanipulator permits this injection apparatus to be mounted directly on three-way movement. With Chambers' micromanipulator it has to be mounted on base beside foot of microscope with a looped, flexible metal tube connecting shaft, *s.*, with micropipette in carrier of micromanipulator. (From *Univ. Calif. Publ. Zool.*, 1925.)

extent. A brass cylinder is fitted with a piece of glass tubing filled with mercury and capped with a solid plug of rubber. The micropipette is cemented into the free end of the glass tube. A metal plunger controlled by the thumb screw pushes against the rubber which in turn controls the column of mercury. The column of mercury acts as a piston in an ordinary syringe. This instrument could be greatly improved by replacing the glass tube with the equivalent of a precision needle holder, thus eliminating the undesirable necessity of cementing the micropipettes to the pipette holder. Dr. McNeil has used this micropipette successfully in isolating and handling the cysts of parasitic amebas.

d. *Fitz's Syringe Micro-Injector-Aspirator*. This instrument (Bausch and Lomb) is made especially for use with the Fitz micromanipulator (shown mounted on micromanipulator in Figure 3). A single control permits either massive injection or microinjection or aspiration. Injections up to 0.5 ml. in volume can be done rapidly by pushing the controlling handle in a pumping fashion. Microinjection is performed by rotation of the control handle. A flexible metal tube connects the syringe to the micropipette holder.

e. *Reyniers' Microinjection Apparatus*.<sup>38</sup> This depends on a method in which air pressure is built up slowly and released instantly which is similar to that formerly recommended by Péterfi. The gas is compressed by the weight of a column of mercury fed through a variable opening. A specially designed push valve is used to allow an instant release of pressure by pressing a button. The push valve is adjustable so that the rate of releasing the pressure may be regulated. The air compression can be built up slowly and may be instantly returned to atmospheric pressure by the push valve. There is no danger of contaminating or diluting the injection fluid. This apparatus was used to form microdroplets in "single cell" isolation experiments. Because of the large volume of air in the system, delicate microinjection of living cells is not possible. For this work the minimum of air space in the system is needed.

f. *Notes on Microinjection Apparatus and Technique*. Both the Taylor and McNeil-Gullberg pipette holders<sup>39</sup> were designed for special micromanipulators. The Chambers microinjection unit may be quickly adapted to any of the existing micromanipulators. In the Taylor-type pipette holder an attempt is made to eliminate an air column between the fluid in the pipette holder and fluid to be injected. The reason for this is obvious.

Because of the extremely small aperture of the microtip, surface tension forces are relatively large. With given back pressures, the fluid may

<sup>38</sup> Reyniers, J. A. *Anat. Rec.*, 56: 307, 1933.

<sup>39</sup> See footnotes 10 and 12.

remain in the tip or it may leak out, depending on the nature of the fluid into which the tip is dipping. In some cases some of the external fluid will enter the pipette even though considerable back pressure is applied. All these possibilities must be considered when quantitative or even qualitative microinjections are demanded.

On the other hand the use of mercury has its disadvantages. Mercury is very easily contaminated, which immediately alters its surface tension characteristics and its cohesiveness. Thus when fluid is to be drawn into the pipette, the mercury column is apt to break, thereby rendering unfit the entire set-up. The use of oil has been tried. Unfortunately oil has the tendency to creep, causing not only the contamination of the injection fluid but also damaging the valve of the instrument. When the bore of the pipette is large (over  $50\mu$ ), mercury may be used.

We have continued working with an air gap separating the injection fluid from the water in the syringe, tube and pipette holder. This set-up is the most expedient when qualitative injections are sufficient. One gradually learns to control the fluid in the microtip by means of the plunger on the syringe. If some fluid leaks out of the tip when introduced into the hanging drop, it is not so serious. Care must be taken so that no fluid from the hanging drop enters the microtip, otherwise one might be unknowingly injecting the hanging drop fluid instead of the desired experimental fluid.

So far no one has devised a microinjection apparatus that will permit quantitative injections. There are two possibilities of approach and it is hoped that in the near future a reliable quantitative micropipette will be developed.

Of the two possibilities for delivering amounts far in excess of microscopic dimensions, one is the Brandt-Rehberg<sup>40</sup> burette which consists of a finely calibrated capillary tube. The expelling and filling mechanism consists of a fine screw which forces a tightly fitting steel piston into a suitable receptacle in the lower part of the burette. This well is filled with mercury. By turning the feed screw the piston displaces the mercury and forces a corresponding amount of mercury into the capillary. The titration fluid occupies the space above the mercury. With this arrangement volumes as small as 0.02 c.mm. can be estimated while the burette is calibrated in 0.2 c.mm. units. The tip of the burette as used by Linderstrøm Lang and Holter<sup>41</sup> has an internal bore of 0.15 mm.

Another type of burette is described by Widmark and Ørskov.<sup>42</sup> A similar screw feed arrangement is provided but the volume is read on a

<sup>40</sup> Brandt-Rehberg, P. *Biochem. J.*, 19: 270, 1925.

<sup>41</sup> Linderstrøm-Lang, K., and Holter, H. *Comp. rend. Lab. Carlsberg*, 19: No. 4, 1931.

<sup>42</sup> Widmark, E. M. P., and Ørskov, S. L. *Biochem. Ztschr.*, 201: 15, 1928.

calibrated micrometer screw instead of on a calibrated burette. In this type possible leakage in the packing surrounding the piston is a likely source of error.

These burettes are filled with mercury and the titration fluid. No air gap exists, thereby providing a precise control of pressure. The future volumetric pipette will in all probability be fashioned after one of these two microburettes. However the difficulties of being able to inject known volumes of the order of  $6.5 \times 10^{-5}$  c.mm. (approximate volume of ameba nucleus) are apparent.

It is possible to inject known amounts of oils or water-immiscible fluids into cells if the droplets become spherical. The diameter of the droplet can be measured with an ocular micrometer and the volume calculated. In some cells, particularly in muscle cells, the oil does not assume a spherical shape but becomes an elongated lenticule. Here volumetric calculations are extremely difficult.

**6. Microinjection Apparatus for Renal Corpuscles, Blood Capillaries, etc.** Wearn and Richards<sup>43</sup> used a Barber pipette holder<sup>44</sup> to hold a sharp-pointed, quartz capillary pipette (point with 10 to  $20\mu$  inner diameter) for withdrawing fluid from the renal capsule of a frog's kidney. The operation is performed in the field of a binocular dissecting microscope. The pipette is connected by means of rubber tubing and a three-way stopcock with a bulb, and the whole apparatus, including the quartz tip, is filled with mercury from the bulb. After inserting the tip of the pipette into the renal capsule the bulb is lowered to create negative pressure sufficient to draw the glomerular fluid into the pipette. For the technique of preparing the frog, arranging the illumination of the kidney and of analyzing the withdrawn fluid the reader is referred to the original article. Dr. Richards has now elaborated a micromanipulator which possesses a thrusting movement which is of great value for these operations.

Keosian<sup>45</sup> used a similar method for removing the cystic fluid from chick mesonephric tubules cultured in vitro. The tonicity of this fluid was measured by the use of a modified Barger method. White and Schmitt<sup>46</sup> used a similar method for the kidney tubules of *Necturus*. They devised a metal pipette holder of comparatively simple construction which permits manipulations sufficiently accurate for their purposes. A full description of it is given in their publication.

<sup>43</sup> Wearn, J. T., and Richards, A. N. *Am. J. Physiol.*, 72: 209, 1924-25.

<sup>44</sup> *Philippine J. Science*, B, 9: 307, 1914. Rev. in *Ztschr. f. wiss. Mikr.*, 32: 82, 1915.

<sup>45</sup> Keosian, J. Ph. D. Thesis, New York University, 1936.

<sup>46</sup> White, H. L., and Schmitt, F. O. *Am. J. Physiol.*, 76: 483, 1926.

Landis<sup>47</sup> has modified Chambers' microinjection apparatus to include a manometer for recording capillary blood pressure and to permit injections under known pressures. When working with the mesentery of a frog a loop of intestine is pulled out and laid loosely over a glass slide. The preparation is kept moist by a constant slow drip of normal saline solution. Florey<sup>48</sup> describes a technique for working with vessels in the mesentery of small animals. This method has been further developed by Zweifach<sup>49</sup> and Baron and Chambers<sup>50</sup> for the purposes of studying the migration of blood cells in frog capillaries.

**7. Darkfield Illumination for Microsurgical Work.** Microdissection and microinjection in the darkfield have been made possible by the use of a special substage condenser of Péterfi<sup>51</sup> manufactured by Zeiss. There are two forms of this condenser, one with a free working distance of 9.4 mm. (N. A. 0.4) to be used only with low power objectives and a second with a working distance of 4.5 mm. (N. A. 0.65) to be used with medium power objectives.

Leitz has developed a darkfield condenser with a working numerical aperture of 0.75-0.80 and with one or two operating openings 11 mm. high through which the microinstruments may be introduced. A special round stage plate 50 mm. in diameter placed over the darkfield condenser serves as a support for the coverslips. This plate also comprises the moist chamber. This equipment gives excellent darkfield illumination.

One may obtain darkfield illumination by using the Ultropak illuminator (see p. 74). The advantages of this equipment include: (1) the ability to use brightfield illumination at will; (2) the wide choice of styles and sizes of moist chambers, and (3) the possibility of using objectives with a numerical aperture of 1.0. The following procedure was used by Kopac<sup>52</sup> in studying the plasmalemma formation in amebas torn with a microneedle. The coverslip containing the amebas in a shallow hanging drop is placed on the moist chamber with the ring condenser on the Ultropak previously adjusted for darkfield illumination. The preliminary adjustments of the microneedles are made in a brightfield as furnished by a substage lamp and condenser. Two switches placed conveniently near the micromanipulator are used to control the two sources of light. At any time, during the time or after the ameba is torn, the

<sup>47</sup> Landis, E. M. *Am. J. Physiol.*, 75: 548, 1925-26.

<sup>48</sup> Florey, H. *Proc. Roy. Soc., S.B.*, 100: 269, 1926.

<sup>49</sup> Zweifach, B. *Anat. Rec.*, 59: 83, 1934.

<sup>50</sup> Baron, H., and Chambers, R. *Am. J. Physiol.*, 114: 700, 1936.

<sup>51</sup> Handb. microbiol. Technik, p. 2471, 1923. Handb. biol. Arbeitsmethoden (Abderhalden), Abt. v, 2: 479.

<sup>52</sup> *Science*, 82: 70, 1935.



substage lamp can be quickly turned off and the darkfield turned on. Thus the effect produced by the microneedles on the protoplasm can be immediately studied with an excellent darkfield illumination.

**8. Microsaltbridges, Microelectrodes and Micromagnets.** In the literature, microinstruments used for establishing electrical contact with protoplasm are frequently referred to as microelectrodes. Blinks<sup>53</sup> pointed out that all non-polarizable so-called microelectrodes should be called microsaltbridges. The capillary point which is to be inserted into a cell is filled with an electrolytic solution and this leads to an electrode of ordinary size. Various types of microsaltbridges and accompanying electrode assemblies have been described. Original papers should be consulted for details.<sup>54</sup> Of those described, the methods of Telkes and Kopac are the most useful. Kopac used an electrode system which combined a Chambers microinjection unit with a calomel half-cell.

Metallic microelectrodes have been described by Taylor<sup>55</sup> and Sen.<sup>56</sup> Taylor constructed his electrodes by drawing a glass of quartz capillary tube containing a metallic wire in a suitable microburner. In preparing these microelectrodes due consideration should be taken of the relative coefficients of expansion of the metal and the glass. The glass or quartz is cut back from the tip (to expose the metal) by a micro-diamond cutter mounted on a micromanipulator and controlled in the field of a microscope. Sen coated the surface of a glass microneedle with metal (1) by silvering using Brashear's process or (2) by sputtering in vacuum. Thicker coats of metal are obtained by electroplating the coated microneedles. However, the metallic surface is frequently apt to rub off, which may introduce a considerable error in the experimental work.

Micromagnets consisting of microneedles with soft iron cores were described by Taylor in the same article.

Various accessories for purposes of measuring the pH of sap and minute quantities of solution have been described by Taylor and Whitaker,<sup>57</sup> and Dorfman.<sup>58</sup> Both of these are micro-hydrogen electrodes. Dorfman's is the most recent and is capable of measuring both the pH and Eh (aerobically) of quantities as small as 0.1 c.mm. Pierce and Mont-

<sup>53</sup> Blinks, L. R. *J. Gen. Physiol.*, 14: 139, 1930.

<sup>54</sup> Ettisch, G., and Péterfi, T. *Arch. f. d. ges. Physiol.*, 208: 467, 1925.

Gefan, S. *Univ. Calif. Publ. Zool.*, 29: 453, 1927.

Telkes, M. *Am. J. Physiol.*, 98: 475, 1931.

Kopac, M. J. *Carnegie Inst. Wash., Papers from Tortugas Lab.*, 29: 361, 1936.

<sup>55</sup> Taylor, C. V. *Proc. Soc. Exp. Biol. & Med.*, 23: 147, 1925.

<sup>56</sup> Sen, B. *Proc. Soc. Exp. Biol. & Med.*, 27: 310, 1930.

<sup>57</sup> Taylor, C. V., and Whitaker, D. M. *Protoplasma*, 3: 1, 1927.

<sup>58</sup> Dorfman, W. A. *Protoplasma*, 25: 465, 1936.

gomery<sup>59</sup> have designed a micro-quinhydrone electrode with which determinations of pH can be made on 0.1 c.mm. fluid or less. This instrument was used by them to determine the pH of glomerular urine of Necturus.

All instruments just described are designed to be mounted on a micromanipulator. The microtips may be kept to the same small limits as those used in the construction of microneedles and micropipettes. They may therefore be controlled with the same degree of precision. For this type of work the Taylor micromanipulator because of its massive construction is the most suitable.

These devices have been used to study the electrical properties of protoplasm (conductivity, electrophoresis, bioelectrical potentials, etc.). Whether the use of these instruments actually eliminates the plasma membrane factor remains for future research to determine.

**9. Additional Accessories.** Titus and Gray<sup>60</sup> have described various microhooks and a microdistillation apparatus for use in conducting chemical experiments on microscopic quantities of matter.

A micro-guillotine designed for breaking the tips of microneedles and micropipettes with considerable precision was described by Belkin.<sup>61</sup>

Péterfi<sup>62</sup> devised a micropincette to be mounted on a micromanipulator. Two microtips are mounted in a special holder. One tip is stationary while the opposing tip may be adjusted in the horizontal and vertical planes by suitable screws. This instrument (Zeiss or Bausch and Lomb) is very useful in handling minute objects but should be equipped with flexible shafts to eliminate the vibrations occurring during manipulation.

Schouten's paper<sup>63</sup> should be consulted for the details regarding the construction and use of microhooks and other ingenious accessories to the micromanipulator.

### III. Applications of the Micrurgical Technique

**1. Protozoology and Embryology.** Rapidly swimming forms can be made temporarily motionless by means of chloretone, magnesium sulphate, urethane, etc. Frequently shreds of cotton or lens paper spread on a coverslip suffice to keep some organisms in a restricted field so that they

<sup>59</sup> Pierce, J. A., and Montgomery, H. *J. Biol. Chem.*, 110: 763, 1935.

<sup>60</sup> Titus, R. W., and Gray, H. LeB. *Ind. Eng. Chem., Anal. Ed.*, 2: 368, 1930.

<sup>61</sup> Belkin, M. *Science*, 68: 137, 1928.

<sup>62</sup> *Handb. microbiol. Technik*, p. 2471, 1923. *Handb. biol. Arbeitsmethoden* (Abderhalden), Abt. v, 2: 479. Instrument supplied by Zeiss or Bausch and Lomb.

<sup>63</sup> Schouten, S. L. *Ztschr. f. wiss. Mikr.*, 51: 421, 1934.

may be seized with the microneedles. Taylor and Farber<sup>64</sup> isolated the ciliate, *Euplotes*, in a shallow hanging droplet of water. The surface tension was sufficient to restrain the movements of the organism. This is suitable only for those organisms possessing a relatively tough pellicle.

Many protozoa and ova may be cut by free hand. However, when the direction of the cut is to be specially oriented or when pieces are to be cut the micrurgical equipment is far more accurate. One needle should be more or less vertical and the other should have its shaftlet bent on itself so that the tip is almost horizontal (p. 85). The vertical needle is to hold the cell and to move it into the desired position. The horizontal needle is the cutting instrument. When cutting a cell in two one must take into account the tendency of many cells to lose their more or less fluid contents if their surface membrane is torn. The use of solutions containing calcium plus a favorable amount of potassium and sodium will aid in the formation of a new membrane at the torn surface.<sup>65</sup> In the cutting process the diagonally directed shaft of the needle is raised into the hanging drop until its tip touches the under surface of the coverslip with its shaft extending under the cell in the direction of the proposed cut. The needle is then raised still farther. Its tip remains more or less in position while the elasticity of the glass allows the shaft immediately back of the tip to rise and cut into the cell as the latter is pressed against the under surface of the coverslip. In this way the ever-deepening constriction in the cell eventually cuts it into two intact pieces. This method has been used in the study of the organization of sea-urchin ova by Tennent, Taylor and Whitaker.<sup>66</sup> For additional details this paper should be consulted.

Nuclei and other cellular organellae may be dissected out or cut with the microneedles. Taylor's<sup>67</sup> study on the neuromotor apparatus of *Euplotes* is a classical example of the use of the micrurgical method in determining the function of cellular organellae.

The following procedure is used in denucleating fresh water amebas.<sup>68</sup>

Two types of glass microneedles are used. One needle used in holding the ameba is of the reflex type (Fig. 15). The shaftlet of the removing needle has only one upward bend. The ameba in a hanging drop on a coverslip is mounted on the moist chamber and brought into the optical field. The holding needle is then raised and brought against the ameba, clamping it against the coverslip.

<sup>64</sup> Univ. Calif. Publ. Zool., 26: 131, 1924.

<sup>65</sup> Costello, D. M. *Protoplasma*, 17: 239, 1932.

<sup>66</sup> Tennent, D. H., et al. *Carnegie Inst. Wash., Papers from Tortugas Lab.*, Publ. 391, 1, 1929.

<sup>67</sup> Taylor, C. V. *Univ. Calif. Publ. Zool.*, 19: 404, 1920.

<sup>68</sup> Holter, H., and Kopac, M. J. In press.

The removing needle is oriented to bring the tip immediately below the nucleus. Then this needle is elevated so that the microtip slightly impales the nucleus. If this step is successful, the nucleus can be removed by moving the impaling needle laterally toward the margin of the amoeba. Very little, if any, cytoplasm is lost provided the removal is done slowly. If the nucleus is removed rapidly, much cytoplasm is lost and in most cases the amoeba will disintegrate completely.

Following the successful removal of the nucleus the holding needle must be gently lowered and the amoeba should be released before the lowering microtip passes through the liquid/air interface of the hanging drop. If the amoeba is only slightly carried past this liquid/air interface complete disintegration may follow. This is particularly true for the delicately membraned *Amoeba dubia*.

Difficulty may be experienced with organisms and ova possessing tough pellicles. In developing ova the investing fertilization membrane can be removed by sucking the still unsegmented eggs into a mouth pipette, the aperture of which is larger than the diameter of the egg but smaller than that of the fertilization membrane. The same result may be obtained by straining the eggs through silk bolting cloth with meshes of the proper size. This removes the fertilization membrane but not other investing pellicles which may be softened by using appropriate salt media. For experimental work on *Arbacia* eggs the reader is referred to an article by Harvey<sup>69</sup> on some of its physical constants.

**2. Cytology and Histology.** During the microdissection of intracellular structures care must be taken to distinguish between the physical state and appearance of the structures before and after death. One must learn to distinguish irreversible injuries from those which are reversible. The observer soon learns to appreciate certain characteristics of the living cell, namely, a peculiar translucency of the cytoplasm and especially of the nucleus. The nucleus is usually the first structure to exhibit visible death changes.

Chromosomes have been obtained for dissection from the pollen mother-cells of certain plants<sup>70</sup> and germ cells<sup>71</sup> of insects. The pollen mother-cells of *Tradescantia* are about  $65\mu$  in diameter. An anther of a young flower bud is crushed between two coverslips in a drop containing equal parts of 10 per cent saccharose and plant sap. The coverslips are then separated and one is inverted over the moist chamber on the stage of the microscope.

The testicular follicles of insects (grasshoppers, crickets, cockroaches)

<sup>69</sup> Harvey, E. N. *Biol. Bull.*, 62: 141, 1932.

<sup>70</sup> Chambers, R., and Sands, H. C. *J. Gen. Physiol.*, 5: 815, 1923.

Wada, B. *Cytologia*, 4: 222, 1933.

Sands, H. C. *J. Gen. Physiol.*, 9: 181, 1925.

<sup>71</sup> Chambers, R. In: Cowdry: *General Cytology*, Chicago, 1924, Sect. v.

are placed in a drop of body fluid or blood serum. A few follicles are then cut off and transferred to a coverslip where they are carefully torn with needles. The germ cells float out free in the drop. The preliminary

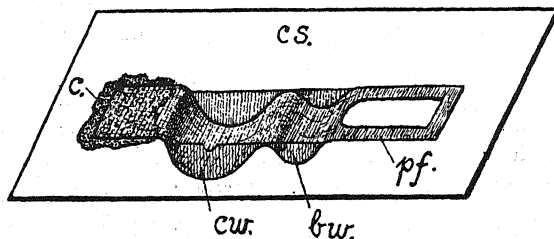


FIG. 17. Celluloid chamber of de Renyi. Celluloid container cemented, at one end, *c*, to undersurface of coverslip, *c.s.*; *c.w.*, chloretone well; *b.w.*, well for body of tadpole; *p.f.*, perforated flap to hold tail against coverslip.

teasing should be done in a moist atmosphere (see moist chamber for teasing, p. 77).

For a discussion regarding artificially made physiological fluids the reader is referred to a review article by Dittler.<sup>72</sup>

An ingenious contrivance for holding a living tadpole in place so that the cellular tissue of its tail can be experimented upon with microneedles has been devised by Dr. Renyi of the University of Pennsylvania (personal communication) (Fig. 17). Strips of thin celluloid are cemented together to form a well held to the coverslip at one end and with an extending horizontal flap at the other. By raising the flap the body of the tadpole can be slipped into the well which holds it snugly with the tail of the tadpole extending flat between the coverslip and the flap of the well. The flap is perforated so that when the coverslip is inverted and mounted on the moist chamber the under surface of the tail is exposed. The microneedles may then be applied to the cells and tissue comprising the tail. An additional well which is in communication with the well containing the body of the tadpole contains a drop of chloretone (1:5000) to narcotize the tadpole.

For streaming protoplasm the unicellular root hairs of *Trianea* or *Hydrocharis* and the stamen hairs of *Tradescantia* are to be recommended. The protoplasts from the epidermis of the onion may be obtained for micrurgical work by using the technique described by Seifriz<sup>73</sup> and Chambers and Höfler.<sup>74</sup>

<sup>72</sup> Dittler, R. In: Abderhalden: Handb. biol. Arbeit. Meth., Abt. v, 1: 379, 1922.

<sup>73</sup> Seifriz, W. *Protoplasma*, 3: 191, 1927.

<sup>74</sup> Chambers, R., and Höfler, K. *Protoplasma*, 12: 338, 1931.

The stretched-out tongue of a pithed frog or the intestinal mesenteries of frogs and small mammals and the mesonephroi of amphibia offer excellent opportunities for micro-operations. Pratt and Reid<sup>75</sup> use the following method for working on the terminal nerve-muscle unit. A disk is made from a glass rod (about 4 by 5 mm.) and cemented with balsam to a glass plate. The tongue of a pithed and narcotized frog is everted and an incision is made through its mid-region as far as the retrolingual membrane. The preparation is adjusted so that the tongue rests on the plate and the disk penetrates the opening thereby supporting the membrane. The preparation is placed in a petri dish and covered with Ringer's solution. Transmitted illumination is used and the tissues in the membrane are manipulated from the top by microelectrodes or microneedles suitably mounted in a micromanipulator.

**3. Tissue Culture.** Cells grown in tissue culture may be readily used in micrurgical work. Several articles have already appeared on results obtained from such studies and recently a detailed description of the special technique necessary has been given by Péterfi.<sup>76</sup> Péterfi discusses the construction of a moist chamber in which manipulations are possible with a maintenance of a constant temperature and a prevention of infection. An adaptation of the Carrel culture tubes to this method is also described.

The micrurgical method is useful in studying the chemotactic properties of various substances in leucocytes grown in vitro. Chambers and Grand<sup>77</sup> used the following method in studying the attraction of leucocytes to sugar. A vertical micropipette, with an aperture of about  $4\mu$  in diameter and containing 5 per cent levulose, was inserted into a hanging drop of chick spleen culture of three to four hours' incubation. Leucocytes, from a maximum distance of about  $100\mu$  were seen, within a few minutes, to turn about and congregate at the tip of the micropipette. Other fluids including the non-aqueous ones may be locally applied to the culture with a micropipette. Solids, starch grains for example, may be inserted into the culture with a microneedle.

**4. Cellular Physiology.** In the field of cellular physiology very important contributions have been made by means of the micrurgical technique. Solutions of various substances can be injected into the cytoplasm, vacuoles or nucleus. Likewise various cellular components may be removed from the living cell.

<sup>75</sup> Pratt, F. H., and Reid, M. A., *Science*, 72: 431, 1930.

<sup>76</sup> Péterfi, T. *Arch. exp. Zellf.*, 4: 165, 1927.

<sup>77</sup> Chambers, R., and Grand, C. G. *J. Cell. Comp. Physiol.*, 8: 1, 1936.

Some reviews of micrurgical and other work on the physical state of protoplasm are those of Weber,<sup>78</sup> Seifriz<sup>79</sup> and Chambers.<sup>80</sup>

The tearing of living cells in various solutions with a microneedle gives considerable knowledge about the formation of a new protoplasmic surface which in many cases behaves similarly to the original protoplasmic surface or the so-called plasma membrane.<sup>81</sup> The "surface precipitation reaction"<sup>82</sup> has been studied in the fresh water amebas.<sup>83</sup> The amebas were torn in solutions containing calcium or strontium. The time necessary for a new surface to be formed was determined by observing its formation under darkfield illumination as furnished by an Ultropak illuminator.

The effect of electrolytes on protoplasm,<sup>84</sup> the pH of protoplasm<sup>85</sup> and the oxidation-reduction potential of protoplasm<sup>86</sup> have been studied by injecting various solutions of electrolytes or dyes into living cells. For experiments of this kind it is essential to have a cell in which moribund changes are easily recognizable. Favorable material includes freshwater amebas, ciliated cells in active movement, active muscle, marine ova and the root and stamen hairs of plants.

So far the pH of protoplasm has been determined colorimetrically. Electrometric methods, although used, require further development. The comparison of color tints of injected cells with standard colors in buffer solutions can be done by projecting the image of the standard colors into the field of the microscope. Pantin<sup>87</sup> describes a good method in which colored test tubes are placed between the source of illumination and the mirror of the microscope. By moving the substage condenser the image of the various colored test tubes can be focussed in the microscopic field. It is possible to use this method also by means of a camera lucida. The Needhams<sup>88</sup> used a set of microtest tubes, that is, glass capillaries filled with dyes and placed on the coverslip in the field of a microscope.

A microcolorimeter devised by Vlès<sup>89</sup> is an apparatus which casts a

<sup>78</sup> Weber, F. In Abderhalden: *Handb. biol. Arbeit. Meth.* 1923, Abt. ii, 2: 655.

<sup>79</sup> Seifriz, W. *Bull. at. Res. Council*, No. 69, 229, 1929. Also in Alexander: *Colloid Chemistry, Theoretical and Applied*. N. Y., 1928, 2: 403.

<sup>80</sup> Chambers, R. In: Harrow and Sherwin's *Textbook of Biochemistry*. N. Y., 1935. Chap. I. Also in Alexander: *Colloid Chemistry, etc.* N. Y., 1928, 2: 467.

<sup>81</sup> Chambers, R., and Reznikoff, P. *J. Gen. Physiol.*, 8: 369, 1926.

<sup>82</sup> Heilbrunn, L. V. *Arch. f. Zellf.*, 4: 246, 1927.

<sup>83</sup> Kopac, M. *J. Science*, 82: 70, 1935.

<sup>84</sup> See articles on micrurgical studies in *J. Gen. Physiol.*, 1926 and after.

<sup>85</sup> Chambers, R., and Reznikoff, P. *J. Gen. Physiol.*, 10: 739, 1927.

<sup>86</sup> Chambers, R. *Bull. Nat. Res. Council*, No. 69, 48, 1929.

<sup>87</sup> Pantin, C. F. A. *Nature*, 111: 81, 1923.

<sup>88</sup> Needham, J., and Needham, D. M. *Proc. Roy. Soc.*, s. B, 99: 383, 1926.

<sup>89</sup> Vlès, F. *Comp. rend. Soc. de biol.*, 94: 879, 1926.

beam of light through a colored filter on a prism mounted in a special ocular (Nachet Cie, Paris). The Reichert photometerocular (C. Reichert, Vienna), a recent development of considerable promise, permits the analysis of the color of the dye injected into a cell by resolving the tint into the percentage content of red, blue and green. This is the additive method of color analysis as contrasted with the spectrophotometric method which is a subtractive method.

Good results are obtained by using a suitable series of pH indicators.<sup>90</sup> Recent work has shown that the potassium salts of the sulphon-phthalein indicators are less toxic than the corresponding sodium salts.<sup>91</sup> The indicators are injected either alone or mixed with a balanced salt solution containing a ratio of K:Na:Ca of 36:4:1. The concentration of the indicator should not exceed 0.4 per cent. Some of the best results have been obtained by several successive injections of a dilute solution of indicator until the first trace of color could be appreciated. A series of nitrophenol indicators<sup>92</sup> is also useful. By injecting living cells with a series of these indicators and noting the color of the dye, a close approximation of the protoplasmic pH may be obtained.<sup>93</sup>

Non-aqueous fluids may be injected into cells with ease. Dawson and Belkin<sup>94</sup> and Marsland<sup>95</sup> injected various oils into the cytoplasm of the fresh-water ameba and respectively determined the digestibility of these oils and their narcotic effect.

By combining the micrurgical technique with the Harvey-Loomis centrifuge microscope<sup>96</sup> some very interesting fields are opened. Harvey

<sup>90</sup> Cohen, B. *Public Health Rep.*, 41: 3051, 1926.

<sup>91</sup> Kopac, M. J. *Carnegie Inst. Wash. Year Book*, No. 34, p. 85, 1934-35.

<sup>92</sup> Michaelis, L., and Gyemant, A. *Biochem. Ztschr.*, 109: 165, 1920.

<sup>93</sup> The following table (see footnote 91) gives the pH values as determined on the mature ova of *Lytechinus*.

Indicator	Color in Cytoplasm	Inferred pH
Brom cresol green . . . . .	Blue	> 5.4
Brom cresol purple . . . . .	Violet	> 6.6
Brom phenol red . . . . .	Red	> 6.6
Chlor phenol red . . . . .	Red	> 6.6
Para nitro phenol . . . . .	Yellow	6.8
Brom thymol blue . . . . .	Green	6.8
Phenol red . . . . .	Yellow	< 6.8
Meta nitro phenol . . . . .	Colorless	< 7.2

The results of this investigation place the cytoplasmic pH of *Lytechinus* between 6.6 and 7.0.

<sup>94</sup> Dawson, J. A., and Belkin, M. *Biol. Bull.*, 56: 80, 1929.

<sup>95</sup> Marsland, D. A. *J. Cell. Comp. Physiol.*, 4: 9, 1933.

<sup>96</sup> Harvey, E. N., and Loomis, A. L. *Science*, 72: 42, 1930. Manufactured by Bausch and Lomb.



and Marsland<sup>97</sup> measured the tension at the surface of the ameba. Their technique, in brief, follows: Olive or paraffin oil droplets of various sizes were injected into an ameba. These injected amebas were placed into the vessels of the centrifuge microscope. By finding the centrifugal acceleration necessary to pull the globule of injected oil out of the ameba, it is possible to calculate the tension of the surface in dynes per centimeter. With similar technique, using instead oils of various densities,<sup>98</sup> it is possible to get important data on the consistency and density of the cytoplasmic matrix.<sup>99</sup>

The microinjection method has produced results bearing on problems of permeability and properties of the so-called plasma membrane. For example, most cells including the ameba and marine ova are impermeable to sulphonphthalein indicators. Yet solutions of these dyes when injected diffuse unimpeded through the cytoplasm and stop at the periphery.<sup>100</sup>

Monné<sup>101</sup> studied the permeability of the ameba nucleus to various dyes. His procedure was to inject solutions of these dyes adjacent to the nucleus. Some dyes penetrated the nucleus while others did not. It is interesting to note that substances toxic to the nucleus of an ameba (*A. dubia*) when injected adjacent to it will cause its immediate ejection.<sup>102</sup>

Brown and Sichel<sup>103</sup> described a method for obtaining the myogram of an isolated skeletal muscle cell. They mounted a single fiber (isolated sartorius muscle fiber in frog serum) upon the tips of two glass needles, one needle being rigid, while the second, the microlever, was flexible so that the tip was free to move when the muscle contracted. The needles, controlled by a micromanipulator, are held in the field of the microscope. By means of a suitable optical system, the image of the microlever was projected on the slit of a recording camera. The muscle cell was stimulated by break induction shocks applied by Ag/AgCl micro-electrodes. Sichel<sup>104</sup> described a method for calibrating the glass micro-levers where it has been determined that the displacement of the tip of the lever is a linear function of the force applied.

**5. Technique of Sub-cooling Cellular Tissues.** The technique of sub-cooling cellular tissues and subsequently inducing the formation of ice crystals within cells is offering a promising field for the detection

<sup>97</sup> Harvey, E. N., and Marsland, D. A. *J. Cell. Comp. Physiol.*, 2: 75, 1932.

<sup>98</sup> n Butyl phthalate has a density of 1.0465.

<sup>99</sup> Work now in progress in this laboratory.

<sup>100</sup> Chambers, R. J. *Gen. Physiol.*, 5: 189, 1922.

<sup>101</sup> Monné, L. *Proc. Soc. Exp. Biol. Med.*, 32: 1197, 1935.

<sup>102</sup> 2,4 Dinitrophenol induces this phenomenon.

<sup>103</sup> Brown, D. E. S., and Sichel, F. J. M. *Science*, 72: 17, 1930.

<sup>104</sup> Sichel, F. J. M. *J. Cell. Comp. Physiol.*, 5: 21, 1934.

of intraprotoplasmic structures. Work of this nature has been done<sup>105</sup> in a room which can be cooled to a temperature at least several degrees below that required in the hanging drop containing the suspension of cells. The cells to be operated upon are mounted on a coverslip in a hanging drop enclosed within a drop of paraffin oil. The coverslip is supported on three or four posts on a slide mounted on the mechanical stage of the microscope with the micromanipulator.

A good method of enclosing the drop with oil is as follows:

A drop of fluid containing the cells is placed on a coverslip and most of the fluid withdrawn with a lip pipette. A ring of paraffin oil is now placed about the drop and a relatively large drop of oil deposited on the center in such a way as to touch the ring and imprison the drop containing the cells. The preparation is now transferred to the low temperature room where the microscopic equipment should have been set up preferably the day before.

A few snow crystals are deposited close to the oil drop and freezing of the drop containing the cells induced by pushing a crystal through the oil into the drop. Freezing of the interior of the cells is induced by inserting the tip of a micropipette previously filled with water and frozen by touching the tip to a snow crystal. A single junction thermocouple may be used for determining the temperature of the hanging drop.

**6. Isolation of Microorganisms.** Among the first to devise mechanical contrivances for isolating bacteria are Schouten,<sup>106</sup> and Barber.<sup>107</sup> It was the Barber's pipette holder which first opened up the possibilities of microdissecting and injecting living cells.

For those who are interested in the technique of isolating bacteria, reference is made to the articles of Schouten, Barber, Kahn,<sup>108</sup> Chambers,<sup>109</sup> and Wright.<sup>110</sup> A more recent review of this technique is that of Reyniers.<sup>111</sup> The first four references deal with the use of a single pipette holder. Wright advocates the use of a double micromanipulator. Wright's procedure, in brief, is as follows:

One pipette is filled with the dilute suspension of the organisms from a young liquid culture. The second pipette is filled with sterile broth. With the first pipette minute droplets are blown on the undersurface of the roof of the moist chamber. As soon as a droplet is found which contains only a single organism the second pipette is immediately brought into the field and the drop-

<sup>105</sup> Chambers, R., and Hale, H. P. *Proc. Roy. Soc. Lond.*, s. B, 110: 336, 1932.

<sup>106</sup> Schouten, S. L. *Konigl. Akad. Wetensch., Amsterd. Proc. Sect. Sc.*, 13: 840, 1911.

<sup>107</sup> *Philippine J. Science*, B, 9: 307, 1914. Rev. in *Ztschr. f. wiss. Mikr.*, 32: 82, 1915.

<sup>108</sup> Kahn, M. C. *J. Inf. Dis.*, 31: 344, 1922.

<sup>109</sup> Chambers, R. *J. Bact.*, 8: 1, 1922.

<sup>110</sup> *J. Lab. & Clin. Med.*, 12: 3, 1927.

<sup>111</sup> Reyniers, J. A. J. *Bact.*, 26: 251, 1933.

let sucked in. This pipette is now removed from the apparatus and the inoculation made on an agar slant or a fluid culture medium.

**7. Additional Applications.** (a) *Colloidal Chemistry.* Freundlich and Seifriz<sup>112</sup> applied the technique of Heilbronn<sup>113</sup> to measure the elasticity of sols and gels:

A very small nickel particle is placed with a micropipette into the substance under investigation. A powerful electromagnet is mounted close to the preparation. By applying a current the pellet is attracted toward the magnet. On release of the current, the particle returns to its original position. The distance traveled by the particle is measured with an ocular micrometer, and if it returns to its original position the distance serves as an index of elasticity. This technique was developed also for determining the elasticity of thin colloidal solutions.

The micrurgical method should lend itself to many problems of colloidal chemistry, particularly those dealing with the properties of surface films, et cetera. For example, Hauser<sup>114</sup> reports the use of the micromanipulator in studying the structure of the latex particle of rubber.

(b) *The Study of Fibers.* Rabinowitsch<sup>115</sup> in studying the microstructure of simple ramie nitrate fibers used a micromanipulator needle to separate the fibrils. Seifriz and Hock<sup>116</sup> by this means have studied the structure of other cellulose fibers.

Single fibers may be studied in polarized light for purposes of determining the change in orientation of the molecular components following stretching. Ramie nitrate fibers treated with a mixture of ethyl malonate and cyclohexanone will swell and thereby show transverse striations which are visible under crossed nicols prisms. Also the isolated fibers may be placed in solvents and other organic media so that swelling and other phenomena may be observed.

(c) *Enzymatic Cytochemistry.* To the enzyme chemist the living cell is an important object of study because (1) the cell is a producer of enzymes and (2) many enzymatic processes take place in the cell. The study of endoenzymes, particularly those of the peptidase group, are of extreme interest. Recently the development of apparatus and methods by Linderstrøm-Lang and Holter<sup>117</sup> has made possible the study of peptidase activity of single cells on a quantitative basis.

<sup>112</sup> Freundlich, H., and Seifriz, W. *Ztschr. physik. Chem.*, 104: 233, 1923.

<sup>113</sup> Heilbronn, A. *Jahrb. wiss. Bot.*, 61: 284, 1922.

<sup>114</sup> Hauser, E. A. *Ind. Eng. Chem.*, 18: 1146, 1926.

<sup>115</sup> Rabinowitsch, B. *Kolloidz.*, 57: 203, 1931.

<sup>116</sup> Seifriz, W., and Hock, C. W. *Paper Trade J.*, 102: 36, 1936.

<sup>117</sup> *Comp. rend. Lab. Carlsberg*, 19: No. 4, 1931.

For the study of this activity on cellular material the following procedure is used:

A small tubular vessel, sealed at one end and of about 250 c.mm. capacity is used for the reaction and titration. Into this is placed 4 to 7 c.mm. of glycerine and water to which is added the material under investigation. After a suitable time (usually thirty to sixty minutes) 7 c.mm. of substrate is added to this. By this time the cell should be completely cytolyzed. The substrate most commonly used is a 0.2 molar solution of alanyl-glycine. The resulting mixture is stirred electromagnetically and placed in a water bath thermostatically controlled to a temperature of 40° c. After twenty-four to forty-eight hours the digestion of the substrate is stopped by adding about 30 c.mm. of 0.05 N HCl in acetone. To this is added 150 c.mm. of an acetone solution of naphthyl red which is used as an indicator. The titration is completed by adding known amounts (with a microburette) of 0.05 N alcoholic HCl until a constant pH value as indicated by the color of the dye is reached. The color standard used has the same dimensions as the reaction vessel. The cleavage of the substrate is determined as cubic millimeters of HCl necessary to complete the titration. For details regarding the pipettes, burettes and apparatus for stirring, the papers by Linderstrøm-Lang and Holter should be consulted.

This new technique may be used to determine the location of various endoenzymes. So far only the peptidase systems have been studied. Many workers are of the opinion that enzymes or the loci of enzymatic activity are closely associated with mitochondria, neutral red staining granules and other cytoplasmic components. In the work of Linderstrøm-Lang,<sup>118</sup> Holter<sup>119</sup> and Holter and Kopac, the evidence seems to indicate that as far as the peptidase system is concerned the activity is not associated with cytoplasmic inclusions but rather with the cytoplasmic matrix.

Holter and Kopac<sup>120</sup> performed the following experiments on amebas to determine what role, if any, the various granules (neutral red and Janus B staining) and crystals play in the peptidase activity. The amebas were transferred to a small centrifuge tube which was previously half filled with a 2 per cent solution of Zulkowsky's soluble starch. The starch solution because of its density serves as a pycnotic cushion for the amebas while they are centrifuged. It was found that stratification of the various inclusions could be accomplished by centrifuging at 2000 r.p.m. for five minutes and then at 7500 r.p.m. for ten minutes. The amebas become essentially ellipsoidal and all granules, crystals and other dense compo-

<sup>118</sup> Linderstrøm-Lang, K. *Comp. rend. Lab. Carlsberg*, 19: No. 13, 1933.

<sup>119</sup> Holter, H. J. *Cell. Comp. Physiol.*, 8: 179, 1936.

<sup>120</sup> See footnote 68.

nents collect at the centrifugal pole. Lipid components collect at the centripetal pole.

Amebas recover rapidly after removal from the centrifuge tube causing a redistribution of the previously stratified cytoplasmic inclusions. Doyle<sup>121</sup> reported that this redistribution is retarded if the amebas are chilled:

A moist chamber was made of duraluminum and designed so that a fluid could be circulated through its walls. A freezing mixture consisting of a concentrated salt solution and crushed ice (temperature about  $-10^{\circ}$  c.) was used to cool the moist chamber. The temperature of the hanging drop was kept near  $0^{\circ}$  c. Immediately after the centrifuge tube containing the amebas was removed from the centrifuge, it was placed in a freezing mixture (temperature about  $-3^{\circ}$  c.). The centrifuged and chilled amebas were transferred to coverslips with a mouth pipette. A coverslip was then placed on the special moist chamber.

This procedure delayed streaming in the ameba for several minutes.

The centrifuged amebas were cut with two microneedles to separate the centrifugal and centripetal portions. The microtips were crossed (initially arranged as in Fig. 15) and resembled a pair of tiny scissors in the microscopic field. The tips and ameba were pressed against the coverslip and by moving the tips longitudinally (a type of scissors action), the ameba was cut. Immediately thereafter a photograph was taken so that a record might be available for volumetric estimations. Then the coverslip was removed from the moist chamber and the two portions were transferred to separate reaction vessels in which 7 c.mm. of phosphate buffer (pH 7.4) was previously placed. One hour later, 7 c.mm. of substrate was added, the contents of each vessel thoroughly mixed and finally the vessels were placed in a water bath at  $40^{\circ}$  c. The digestion period varied from eighteen to twenty-four hours. The amount of splitting of the substrate was determined by titration (see p. 107).

Mr. C. G. Grand of this laboratory has taken a large part in developing and improving the micrurgical technique, and we appreciate the unselfish interest of Mr. Henri Dumur, Mr. Ludwig Leitz and Professor M. Berek of Wetzlar whose engineering resources have made possible the development of the various optical and mechanical instruments necessary in our work.

<sup>121</sup> Doyle, W. L. *Collecting Net*, 8: 80, 1933.

TABLE I

Taper	Size	Diameter of Shaftlet	Diameter of Microtip at Base	Length of Microtip	Diameter of Opened Tip
Quick	Coarse	35 $\mu$	8 $\mu$	60 $\mu$	2-5 $\mu$
	Medium	10-15 $\mu$	3 $\mu$	35 $\mu$	$\frac{1}{2}$ -2 $\mu$
	Slender	6-10 $\mu$	2 $\mu$	10-20 $\mu$	$\frac{1}{2}$ -1 $\mu$
Gradual		Diameter of Shaftlet 30 $\mu$ from Microtip			
	Coarse	5 $\mu$			
	Medium	3 $\mu$			
	Slender	2 $\mu$			

Table giving actual sizes of the two serviceable types of microneedles, the quick taper (cf. Fig. 12 c) and the gradual taper (cf. Fig. 12 d), classified as coarse, medium and slender.

# CHEMICAL AGENTS: VITAL STAINS

NATHAN CHANDLER FOOT, M.D.

Introduction 110. Routes of injection 110. Types of material used 111. Specificity of vital dyes 111. Applicability of vital staining 112. Technique of vital staining 113. Fixation of vitally stained tissues 116. Combinations of vital stains 116.

## I. Introduction

The term "vital staining" is somewhat misleading; "vital" it certainly is, being performed *intra vitam*, but it is hardly "staining" in the usual sense of that word. If the reader expects to obtain diffuse cytoplasmic and precise nuclear coloration by this method, as one would with the more usual technique, he will be disappointed. Vital staining applies to a specific coloration of the cytoplasm of certain cells, chiefly those of the "reticuloendothelial system" and, so far as we are concerned, consists of a process of ingestion of particles, either solid or colloidal, by these cells. There has been much speculation as to how the cytoplasm becomes vitally stained. Hypotheses have been advanced in favor of a specific affinity of preexisting granules in the cytoplasm for these dyes, of the filling of vacuoles by the particles of coloring matter, or of a simple process of phagocytosis of particulate or colloid material. It is unimportant to us which of these is the true solution of the problem, although the last two are probably nearer the truth than is the first.

In employing vital stains, the syringe and needle are the only apparatus needed. Naturally, aseptic technique is imperative. In using these stains intravenously an added precaution is necessary since one must guard against the presence of particles, or clumps of coloring matter large enough to form emboli in the capillary vessels. The use of normal salt solution as a suspending fluid is usually absolutely interdicted, particularly if benzydine dyes or other colloidal suspensions are to be employed, because it clumps the particles and brings about vascular occlusion with results that are usually as prompt as they are fatal.

## II. Routes of Injection

Vital dyes may be injected into any part of the body, depending upon what is desired; they are usually used: 1. **Intravenously**, 2. **Intraperitoneally**, or 3. **Subcutaneously**. The dosage in the last two methods is of less importance than in the first; rather large doses are necessary to

bring about complete results in their case, while smaller doses are needed with intravenous administration. These are the common routes, but vital dyes may be injected into other cavities or systems of the body if so desired, such, for instance, as the pleural sac or the trachea.

### III. Types of Materials Used

The substances used for vital staining may be divided into four chief classes:

1. **Simple suspensions of particulate material**, such as lampblack, India ink, carmine, indigo carmine, graphite, metals, etc.
2. **Colloidal suspensions of similar substances**, Higgins' ink, lamp-black and gelatin, lithium carmine, etc.
3. **Colloidal suspensions of silver or gold**, or their salts, particularly albuminates such as protargol, argyrol.
4. **Colloidal suspensions of various benzidine dyes**, such as trypan blue, Niagara blue, isamin blue, pyrrhol blue, trypan red, Janus green, etc.

The choice of one of these depends largely upon the desire of the experimenter and the nature of the experiment to be performed. Sometimes one method, sometimes another, will prove most satisfactory; they may often be combined, as in the instance of experiments to determine the origin of the "dust cells" of the lung, where one dye was injected into the trachea and the other through the blood stream.<sup>1</sup> Sometimes it is merely a question of convenience, some dyes being readily available, while some are not.

### IV. Specificity of Vital Dyes

1. **Phagocytes.** If one desires to stain the phagocytes, almost any one of the above mentioned materials may be used, injected either directly into the immediate vicinity of the cells, or into the peritoneum, pleural cavity, or vascular system. The phagocytes comprise several types of cells that have been given a variety of names, according to their morphology—endothelial leucocytes, monocytes, clasmatocytes, histiocytes, etc., etc.

2. **Leucocytes.** Many of the dyes (particularly colloidal carbon) will, when injected intravenously be taken up by the monocytes; the polymorphonuclear leucocytes rarely ingest any but the benzidine dyes and they do this usually only under circumstances indicating stagnation

<sup>1</sup> Permar, H. H. *J. Med. Research*, 52: 9, 1920.

Foot, N. C. *J. Exper. Med.*, 32: 533, 1920.



of the circulation. They are sometimes seen stained in thrombi or in pneumonic exudates, for example. In this instance, however, the dye stains the granules of the leucocytes quite brilliantly, which points to a difference in the staining mechanism and suggests that its entrance into these cells is not purely a matter of ingestion. Lymphocytes and erythrocytes do not stain with these dyes.

3. **Connective Tissue.** The fibroblasts are sometimes vitally stained, taking up most of the materials enumerated if given sufficient time. There are various ways of explaining this, but they would lead us far afield.

4. **Vascular Endothelium.** As a rule, this is not stained by the dyes we have already discussed, with the exception of colloidal carbon in suspension, as in Higgins' ink. Sometimes, in cases of stagnation, it may become colored by other materials, but this is unusual. Inflamed vascular endothelium is regularly impregnated by colloidal carbon and this fact is often overlooked; swollen, inflamed endothelium probably becomes sticky and the carbon particles adhere to its surface and gradually become taken up by the cytoplasm. The Kupffer cells are regularly stained with all of the vital dyes, a fact that tends to support the contention that they differ somewhat from ordinary vascular endothelium.

5. **Epithelial Cells.** Vital stains sometimes enter epithelial cells to a rather marked extent. If the staining be pushed beyond certain limits almost any of these substances will find their way into the liver cords. The benzidine dyes and lithium carmine, which are regularly excreted through the kidneys, stain the epithelium of the convoluted tubules. Otherwise the vital dyes do not appear in epithelium with any regularity.

*Other Tissues.* The various tissues of the body not already enumerated, do not become stained with these pigments or dyes.

## V. Applicability of Vital Staining

This method is of the greatest value in investigating the phagocytic cells of the body, wherever they may be situated; the reticuloendothelium is readily identified by means of lithium carmine or the benzidine dyes, as are the histiocytes, or wandering connective tissue cells. The vascular endothelium may be investigated and identified by means of colloidal suspensions of carbon. The excretory and absorptive areas of the kidney epithelium may be mapped out by employing lithium carmine.<sup>2</sup> As the bone marrow contains such reticuloendothelium and many phagocytic cells, it may be vitally impregnated with colloidal carbon, become quite black and opaque, and its distribution in cancellous tissue be studied in

<sup>2</sup> Suzuki, T. *Nierensekretion*, Jena, 1912.

three dimensions by clearing the bone by the Spalteholz method.<sup>3</sup> The benzidine dyes may be used in embryology to study the absorption of the amniotic, or other fluids, by introducing them into the proper sacs.<sup>4</sup>

## VI. The Technique of Vital Staining

1. **Particulate Matter.** Various particulate substances and pigments may be suspended in distilled water, filtered through coarse filter paper to remove the coarser granules, sterilized and used for injection into the vascular system, body cavities, or subcutaneous tissue. Heidenhain (Schmorl cit., 1921)<sup>5</sup> recommended a 0.4 per cent suspension of indigo carmine for intravenous injection, 35 c.c. to 60 c.c. being administered to rabbits, 150 c.c. to 1500 c.c. to dogs, according to their size. For intraperitoneal, intrapleural or subcutaneous injection the amount depends upon the size and species of the animal used and should be determined by experimentation. Karsner and Swanbeck<sup>6</sup> used suspensions of carmine or lampblack for intrapleural injection in cats in amounts varying from 15 c.c. to 25 c.c. For subcutaneous injection the capacity of the areolar tissue to take up the dye is the best indication of the amount to be used, and this varies with the location—very little can be injected into the external surface of a rabbit's ear, but a good deal can be injected under the loose skin of the back. Any insoluble pigment may be used. Slavjansky<sup>7</sup> employed double injections (vascular and intratracheal) of particulate indigo and cinnabar as early as 1869.

Drinker and Shaw<sup>8</sup> have introduced a method of using suspensions of fine particles of manganese dioxide in acacia water for investigating the phagocytic power of the endothelium of various organs in different animals; the manganese is injected and then each organ is assayed about one hour later, to determine its manganese content. The particles may be seen under the microscope, in the endothelial cells, but this is a chemical test, rather than a staining method and the reader is referred to their article for further details.

2. **Colloidal Suspensions of Particulate Matter.** Particulate matter may be rendered colloidal by suspension in solutions of acacia or gelatin, when it becomes more precise in its action and acquires an affinity

<sup>3</sup> Wislocki, G. B. *Bull. Johns Hopkins Hosp.*, 32: 132, 1921.

<sup>4</sup> Wislocki, G. B. *Bull. Johns Hopkins Hosp.*, 32: 93, 1921.

<sup>5</sup> Schmorl, G. *Die pathologisch-histologischen Untersuchungsmethoden*, Leipzig, Ed. 10-11, 1921.

<sup>6</sup> Karsner, H. T., and Swanbeck, C. E. *J. Med. Research*, 42: 91, 1920.

<sup>7</sup> Slavjansky, K. *Virchow's Archiv. f. path. Anat.*, 48: 326, 1869.

<sup>8</sup> Drinker, C. K., and Shaw, L. A. *J. Exper. Med.*, 33: 243, 1921.

for the monocytes and vascular endothelium. This is particularly true of colloidal carbon because particulate carbon, unaided by any emulsifying agent, is quite unsafe for this purpose. This should always be borne in mind.

*Higgins' Ink.* Higgins' waterproof black drawing ink, which is (in all probability) a suspension of finely-divided carbon in acacia water, with a little camphor as a preservative, is the most readily available colloidal carbon for laboratory use. It is necessary merely to dilute it with an equal volume of sterile distilled water in a sterile test-tube and it is ready for use. It is well to warm it to body temperature before injecting it. The average intravenous dose for rabbits is 5 c.c., repeating this daily for three or four days and then every three days thereafter. The ear is shaved, rubbed with alcohol and a marginal vein used as the most accessible site for injection. If the vein be too small to be entered readily by the needle, a little xylol rubbed over the skin will cause the veins to become congested and easily entered; of course, a little practice is necessary. Injections should be begun near the tip of the ear and each subsequent injection should be given a short distance nearer the head, and the direction of the blood stream; this makes it possible to use the same vein several times before it becomes thrombosed. A sharp needle, introduced as short a distance as possible into the lumen of the vein, is an added precaution against thrombosis; do not injure the endothelium if it can be avoided. The use of this ink was introduced by George Wislocki of the Johns Hopkins Medical School, where it has been in use by various investigators.

*Colloidal Lampblack.* If one desires to make up colloidal carbon suspensions in the laboratory, this may be done by McJunkin's method.<sup>9</sup> Grind up a good quality of commercial lampblack in a mortar for half an hour and add 0.4 gm. of this to 100 c.c. of distilled water that contains 2 per cent gelatin. Sterilize and use as in the case of Higgins' ink. The gelatin may be used in 1 per cent suspension and a small quantity of this added to 5 gm. of lampblack, little by little as the latter is ground to a fine paste (Simpson, 1922<sup>10</sup>). This is then made up to 100 c.c. with 1 per cent gelatin suspension. McJunkin advises administering 5 c.c. to 9 c.c. of 10 per cent sodium citrate solution at the same time the carbon is injected, claiming that he thus produces an added specificity and affinity of the monocytes for the carbon.

Both of these methods are specific for monocytes and for vascular endothelium, particularly in inflamed areas. Ordinary particulate suspensions will not be taken up in the same way. It has been noted by

<sup>9</sup> McJunkin, F. A. *Arch. Int. Med.*, 21: 59, 1918.

<sup>10</sup> Simpson, M. J. *J. Med. Research*, 43: 77, 1922.

Lang that colloidal suspensions of carbon become agglutinated when mixed with blood, thus becoming "common suspension of rather coarse carbon particles." Be this as it may, the effects of injected particulate carbon are quite different from those of colloidal carbon. For a full discussion of this subject, the reader is referred to papers by Lang<sup>11</sup> and Wislocki.<sup>12</sup>

*Lithium Carmine.* This is a colloidal suspension of carmine, first devised by Ribbert (Schmorl, p. 113) for studying kidney function, and largely employed and popularized by Suzuki (p. 112), Aschoff and Kiyono,<sup>13</sup> and Kiyono.<sup>14</sup> A concentrated suspension of carmine rubrum optimum (5 gm.) in cold saturated lithium carbonate is filtered and sterilized and 5 c.c. to 10 c.c. injected intravenously, in the case of rabbits. It must be slowly administered. It is well tolerated by most animals, but has been found very toxic in one species, the Japanese waltzing mouse and its hybrids. This should be taken into account if that animal is the subject of experimentation.

**3. Colloidal Suspensions of Silver and Gold.** Protargol was used intravenously by Askanazy,<sup>15</sup> Miller,<sup>16</sup> and others at one time. Simpson has employed "red gold" (40 per cent gold in 60 per cent sodium lysalbinat), for staining the monocytes and Kupffer cells. These substances present no advantage over the other methods and have not gained enough popularity to warrant a discussion or description in this section. References have been supplied.

**4. Colloidal Suspension of Benzidine Dyes.** Vital staining with these substances was first discovered by Goldmann,<sup>17</sup> who obtained them from Ehrlich's laboratory. They were introduced into the United States by H. M. Evans<sup>18</sup> who employed them in a number of investigations. Their chemistry is fully discussed in an article by Evans and Schulemann.<sup>19</sup>

As already stated, there are a number of these dyes, most of which are more or less toxic, so that it is best to limit oneself to those of proved worth, namely trypan blue and its American prototype Niagara blue 2b. (N.A.C. Co. Buffalo, N. Y.), both of which are as nearly innocuous as foreign material introduced into the circulation could be.

<sup>11</sup> Lang, F. J. *Arch. Pathol.*, 1: 41, 1926.

<sup>12</sup> Wislocki, G. B. *Am. J. Anat.*, 32: 423, 1924.

<sup>13</sup> Aschoff, L., and Kiyono, K. *Folia Haematol*, 6: 213, 1913.

<sup>14</sup> Kiyono, K. *Die vitale Karminspeicherung*, Jena, 1914.

<sup>15</sup> Askanazy, M. *Aschoff's Pathologische Anatomie*, Jena, 1923, 1, 183.

<sup>16</sup> Miller, J. *Beitr. z. path. Anat. u. z. allg. Path.*, 31: 347, 1902.

<sup>17</sup> Goldmann, E. E. *Beitr. z. klin. Chir.*, 44: 192, 1909; 78: 1, 1912.

<sup>18</sup> Evans, H. M. *Am. J. Physiol.*, 37: 243, 1915.

<sup>19</sup> Evans, H. M., and Schulemann, W. *Science*, N. Y., n.s., 39: 443, 1914.

For use, they are dissolved (suspended) in sterile distilled water, 1 gm. per 100 c.c. and used immediately, for it is unsafe to use electrolytes, such as normal saline or Ringer's solution, and it is dangerous to allow the suspensions to stand for more than an hour or so, for they are apt to become agglutinated and become highly toxic. They may be used intravenously, subcutaneously, or injected into body cavities; the dosage for subcutaneous administration in rabbits, is 1 c.c. per 20 gm. body weight; for intraperitoneal injection, 10 c.c. to 15 c.c.; for intravenous administration, 5 c.c. to 10 c.c., slowly injected. The suspension should be brought to body temperature before injection. The day following the injection the skin takes on a distinctly bluish tinge and, if the injections be repeated several times, the animal becomes quite blue, skin and mucous membranes having become deeply stained, and excretes bright greenish-blue urine. If a red dye be preferred, lithium carmine may be used, or trypan red; but the latter is distinctly more toxic than trypan blue and should be administered at half the concentration of that dye.

## VII. Fixation of Vially Stained Tissues

For material stained with particulate material and the colloidal suspensions of carbon or metals, any fixation is appropriate, but if lithium carmine or the benzidine dyes have been used, they will lose in intensity if Zenker's fluid be employed for fixation, on account of the acetic acid it contains. Neutral 10 per cent formalin (4 per cent formaldehyde) undoubtedly gives the best results. If a chromium fixative be desired, however, Helly's solution (Müller's fluid 90 per cent, strong neutral—40 per cent formaldehyde—formalin 10 per cent) gives excellent results, although there is some paling of the blue dyes which become slightly greenish. The material is most satisfactorily cut in paraffin, although frozen sections or celloidin sections give good results.

## VIII. Combinations of Vital Stains

As might be supposed, it is possible to combine two or more types of vital stains if so desired. For example, one may inject colloidal carbon intravenously and administer Niagara blue intraperitoneally at the same time, using lithium carmine intrapleurally if so indicated. Some cells will ingest one dye, some another and others (the endothelial phagocytes, for example) may take up all three. The histiocytes or reticulo-endothelial cells tend to take up the benzidine dyes and lithium carmine quite early, the carbon appearing later; or, a dye of one color may be administered intravenously and one of another hue given intratracheally, for experiments on the pulmonary circulation, etc. The experimenter may thus devise a number of useful combinations.

## CHEMICAL AGENTS: SUPRAVITAL STAINS

FLORENCE R. SABIN

Supravital dyes 117. Methods of staining 118. Applications of supravital stains 122.

### I. Supravital Dyes

The supravital technique is based on the discovery that certain dyes penetrate living cells and stain specific materials within them. These dyes enter cells in solution and thus are not phagocytosed as particulate matter. They are therefore not like the so-called *vital dyes*, which, when injected into the living animal, demonstrate the power of certain cells to phagocytose particles. However, in as much as some of the dyes used in the supravital reaction are segregated by the cell into its vacuoles of digestion these dyes may also be used to study the activities of phagocytic cells. A prime essential for the supravital dyes is that they should not damage cells appreciably in the concentrations used. When cells are killed by these dyes, both nucleus and cytoplasm are stained alike and uniformly and hence the result is much less distinctive than that following the use of dyes on fixed tissues.

Of the dyes used for supravital staining there are two groups; the first, of which *vital neutral red*<sup>1</sup> is a type, reacts to several different kinds of substances within cells; the second, of which *Janus green* is representative, responds to one substance only. Of all the dyes so far tested, neutral red and Janus green have proved to be the most useful. Neutral red is the least toxic of all and has the advantage of being a chemical indicator within certain limits. Its color toward the acid end of its range, about pH 6, is a scarlet red; while toward the alkaline end, it becomes orange, and at pH 8 is a clear yellow.

1. **Dyes Reacting to Several Substances.** In this group, there are a number of different dyes, all of them weak bases,<sup>2</sup> *methylene blue GG*, *azure*, *brilliant cresyl blue*, and *neutral red*. A supravital dye can be made by diluting the *Wright's* methylene-blue-azure used for staining fixed films of blood and obtaining from it a dry film. It is, however, less useful than neutral red.

<sup>1</sup> Conn, H. J. *Biological Stains*. Geneva, N. Y., 1925.

Phillips, M., and Cohen, B. *Stain Technol.*, 2: 17, 1927.

<sup>2</sup> Irwin, M. J. *Gen. Physiol.*, 10: 75, 1926-27.

It was shown by Campbell<sup>3</sup> in 1886-88 that the nuclei of certain plant cells could be stained supravitally with *mauvein*, *methyl violet* and *dahlia* without interfering with cell division or with cytoplasmic streaming; but with the cells of animals, staining of the nucleus or of the basic cytoplasm with any of the dyes yet known must be taken as an indication of too great a damage to the cell and as vitiating the method.

Neutral red reacts to four different types of intracellular materials: First, certain granulations which are the products of cytoplasm. These are the specific granulations of the white blood cells, neutrophilic, basophilic, eosinophilic, pseudoeosinophilic or heterophilic, and amphophilic, together with certain of the secretory granules, for example those of the islet cells of the pancreas.<sup>4</sup> Second, this dye is concentrated by the phagocytic cells into the vacuoles of digestion. Within these vacuoles some of the dye may become absorbed by the phagocytosed material. Third, the dye demonstrates the canalicular apparatus of the parietal cells of the gastric mucosa.<sup>5</sup> Fourth, the basophilic substance of young red cells is precipitated by neutral red, as well as by brilliant cresyl blue, into the so-called reticulation.

2. **Dyes Reacting to One Substance.** Of the dyes of the second group, *vital Janus green*, which stains mitochondria only in the living cell, is the best known. Bensley (1911-12) has shown that *pyronin*, 1 to 1000, is specific to bring out the blind ducts of the islets of the pancreas and that methylene blue, injected in the concentration of 1 to 10,000 into the blood vessels of the pancreas, brings out characteristically the centroacinar cells. The number of these specific vital reactions of special dyes will undoubtedly be greatly increased with further experimentation.

## II. Methods of Staining

1. **Preparation of Dry Color Films.** Supravital staining of living cells can be obtained in two ways, either by placing cells on slides which have a dry film of stain upon them, in which case the stain is dissolved by the natural fluids which surround the cells, or by dissolving the dyes in physiological saline and injecting the solution into the blood vessels of an animal under ether or immediately after death. Both methods have their specific uses.

Dry films of brilliant cresyl blue were used by Levaditi<sup>6</sup> in 1901, but in concentrations that killed the cells. The technique of the dry

<sup>3</sup> Campbell, D. H. *Untersuch. a. d. botanischen Inst. zu Tübingen*, 2: 569, 1886-88.

<sup>4</sup> Bensley, R. R. *Am. J. Anat.*, 12: 297, 1911-12.

<sup>5</sup> Harvey, B. C. H., and Bensley, R. R. *Biol. Bull.*, 23: 225, 1912.

<sup>6</sup> Levaditi, C. *J. d. physiol. e. de path. gén.*, 3: 424, 1901.

color films for true supravital staining of cells was fully developed by Simpson<sup>7</sup> in 1921, and by Sabin<sup>8</sup> in 1923. The method was also described by Forkner<sup>9</sup> in 1930. They used both vital neutral red alone and neutral red in combination with Janus green. This technique of dry films of stain on the slide on which cells are to be placed has the advantage of allowing the cells to be studied in their own fluids.

The first requisite is to have stains which are true vital dyes. In 1927, Dr. Max Phillips and Dr. Barnett Cohen,<sup>10</sup> of the Hygienic Laboratory, United States Public Health Service, Washington, D. C., published a study of the methods of preparing *neutral red iodide*, which is the preparation of this dye used for supravital staining. The *Janus green* which stains mitochondria in living cells is *diethylsafranin*, as shown by Cowdry.<sup>11</sup> In as much as cells are sensitive indicators of variations in vital dyes, it is necessary that each sample of the dye manufactured should be carefully tested and this matter has been taken over by the Commission on Standardization of Biological Stains, of which Dr. H. J. Conn<sup>12</sup> of the Agricultural Experiment Station, Geneva, New York, is the Chairman. Under his direction, samples of both neutral red and of Janus green to be used as vital dyes are tested against living cells before they are put on the market.<sup>13</sup> In buying these dyes it should be ascertained that they have been so certified.

The stains used for the *dry film method* are dissolved in absolute alcohol, flooded onto the slides, and left as a dry film by a complete evaporation of the alcohol. The slightest acidity in the alcohol makes the dyes worthless for the staining of living cells; the alcohol must therefore have been distilled from lime and the neglect of this precaution has been responsible for many failures with the technique.

The solutions to be used for making the films are so dilute that it is convenient to make them from stock saturated solutions, in order to avoid weighing out very small quantities. We use 125 mg. of the neutral red iodide to 50 c.c. of neutral absolute alcohol, and 125 mg. of the Janus green to 62.5 c.c. of the alcohol. In this strength there will be a

<sup>7</sup> Simpson, M. E. *Univ. Cal. Pub. in Anat.*, 1: 1, 1921. *Anat. Rec., Proc. Amer. Assn. Anat.*, 21: 82, 1921.

<sup>8</sup> Sabin, F. R. *Bull. Johns Hopkins Hosp.*, 34: 277, 1923.

<sup>9</sup> Forkner, C. E. *J. Exp. Med.*, 52: 379, 1930.

<sup>10</sup> Phillips, M., and Cohen, B. *Stain Technol.*, 2: 17, 1927.

<sup>11</sup> Cowdry, E. V. *Carnegie Inst. of Washington, Pub. No. 271. Contrib. to Embryol.*, 8: 39, 1918.

<sup>12</sup> Conn, H. J. *Stain Technology*, 9: 81, 1934.

<sup>13</sup> Such certified stains can be obtained from the National Aniline and Chemical Company, New York City. Hynson and Westcott, Baltimore, Md., also supply certified vital stains.



few granules of the dye on the bottom of the bottles and care must be used not to stir them into the solution. These solutions should be kept in glass-stoppered bottles, preferably stoppered with glass pipettes. The utmost care must be used not to let the dye run into the rubber nipple.

The dilute solutions should be made only in small quantities, not more than 5 or 10 c.c. at a time, for the slightest dust or grease introduced will spoil the whole sample. To make the dilute neutral red solution, add 1.1 c.c. of the saturated solution to 10 c.c. of neutral absolute alcohol. To make the mixture of neutral red and Janus green, add 0.04 c.c. of the saturated solution of the Janus green to 3 c.c. of the dilute neutral red solution. The combination of the neutral red and Janus green is not stable in solution and so should be put onto slides the day it is made up.

The slides for the dry films should be chemically clean, without a trace of grease and entirely free from dust. To prepare the films, hold the slide obliquely over the bottle of the stain, flood its surface evenly with dye and let the excess run back into the bottle. Avoid letting the dye touch the fingers. To remove the last traces of excess dye from the slide, touch its edges to an absorbing surface, such as cheese cloth or a blotter, and put the slide horizontal in a current of warm air to dry; this can be induced by having a lighted Bunsen burner in a horizontal position nearby. No streaks of dye should be visible on the slides, for such concentrations kill the cells. The stained surface of the slide should be marked with a glass pencil and the slides protected from dust. The optimum amount of dye depends on the number of cells in the preparation and on this account some variations in concentration must be made to adapt the method to different problems. In general the concentrations just given are adequate for blood films, but for other purposes double the amounts given may be used. Three different preparations will be found useful for specific purposes, the *neutral red* alone, the *Janus green* alone, and the combination of the two dyes. In general the combination of *neutral red* and *Janus green* is the most used. The Janus green is much more toxic than the neutral red and inhibits the motility of the cells. Thus for specimens of blood in which motility is important, as for example in the discrimination of the B-myelocytes from monocytes for the diagnosis of monocytic leucemia, the films of the neutral red alone should be used. In general the amount of the Janus green in the double dye preparations should be cut to the very minimum necessary to bring out the mitochondria.

**2. Vascular Injection of Dyes to be Used for Supravital Reactions.** The use of supravital staining of cells by injecting the dyes dis-

solved in physiological saline has been developed especially by Bensley<sup>14</sup> and by Harvey and Bensley.<sup>15</sup> Neutral red in the strength of 1 to 10,000 and Janus green, 1 to 15,000, can be injected into the blood vessels immediately after killing the animal. Many dyes valuable for this technique are already known. In making the injections the vascular route is the most used, but such dyes may be introduced interstitially or into the body cavities. The technique for the vascular injections is described in the next section.

**3. Fixation of Supravital Dyes.** The range of the use of the supravital reaction was much extended by showing that this staining of the cells could be preserved in fixed tissue. This was first brought out by Sherwood<sup>16</sup> who developed a method for fixing films of cells from exudates which had been supravitaly stained. She used a buffered *Ringer-formalin solution* as the fixative. Methods were then devised for preserving the supravital reaction in organs, followed by embedding in paraffin. These methods were developed by McJunkin, Cash, Gardner and Forkner.<sup>17</sup> McJunkin obtained the supravital reaction by *interstitial injection* of the dye and fixed the tissues in Helly's fluid, which is Zenker-formol. The prime essential of this technique is, however, to obtain perfect supravital staining and this is done much better by vascular injection of the dye. Forkner has modified the methods of Cash and described them in detail. The essentials are: three flasks, one for pressure connected with a manometer; a second to contain the warm neutral red solution (1000 c.c. of 0.6 per cent vital neutral red in 0.9 per cent saline); and the third to contain 500 c.c. of warm Zenker base with 15 per cent neutral formol. Instead of tying a cannula into the heart or the aorta, he found that it saved time to introduce a small fish hook into the injection needle; the fluid could flow around the hook easily and it was adequate to prevent the needle from coming out of the vessel. The apparatus was fitted with a three-way cock so that the fixing fluid could be turned on immediately when the desired staining had been obtained. The perfusion with the stain should last from fifteen to twenty minutes, and the fixing fluid should run about ten to twenty minutes. The organs are then placed in the fixing fluid for twelve to twenty-four hours; small blocks, not over 2 mm. in thickness, are then

<sup>14</sup> Bensley, R. R. *Am. J. Anat.*, 12: 297, 1911-12.

<sup>15</sup> Harvey, B. C. H., and Bensley, R. R. *Biol. Bull.*, 23: 225, 1912.

<sup>16</sup> Sherwood, M. B. *Proc. Soc. Exp. Biol. & Med.*, 23: 622, 1925-26.

<sup>17</sup> McJunkin, F. A. *Am. J. Path.*, 1: 305, 1925.

Cash, J. R. *Proc. Soc. Exp. Biol. & Med.*, 24: 193, 1926-27.

Gardner, L. U. *Proc. Soc. Exp. Biol. & Med.*, 24: 646, 1926-27.

Forkner, C. E. *J. Exp. Med.*, 52: 379, 1930.

run rapidly through the following solutions, blotting the excess of fluid between each transfer.

Solution 1	Absolute ethyl alcohol.....	1 minute
2	Absolute ethyl alcohol.....	5 minutes
3	Pure acetone.....	20 minutes
4	Pure acetone.....	20 minutes
5	Pure acetone.....	20 minutes
6	Xylol.....	20 minutes
7	Xylol.....	20 minutes
8	56° c. Paraffin.....	1-2 hours
9	56° c. Paraffin.....	1-2 hours
10	Embed	

For a counterstain, Goodpasture's<sup>18</sup> acid polychrome methylene blue gives excellent results; the dehydration and clearing must be done as rapidly as possible. Another way of treating the sections is to use the Foot and Mènard's<sup>19</sup> technique for staining reticulum by silver impregnation. If the bleaching of this technique be omitted, the neutral red staining will be replaced by the silver and every detail of the supravital reaction will be reproduced.

### III. Applications of Supravital Staining

#### 1. *Blood*

The most extensive use of the supravital reaction has been applied to the study of the cells of the blood. In general the supravital technique brings out evidences of functional changes in the cytoplasm better than fixed films; nuclear structure, though visible in the living cell, is demonstrated better in fixed tissues. Thus a differential for an Arneth-Schilling count is made more rapidly and perhaps more accurately with fixed films. The supravital technique is more sharply differential for the monocyte than other methods; this is more true for the monocytes in the blood of animals than in human blood. The monocyte in human blood contains characteristic granules brought out in fixed films and giving this cell a positive oxidase reaction. In the blood of all animals, including the monkey, these granules are lacking and the cell is oxidase-negative.

For preparations of blood the double stain, neutral red and Janus green, is the method of choice. The Janus green will inhibit the motility

<sup>18</sup> See Mallory, F. B., and Wright, J. H. *Pathological Technique*. Ed. 8, Phila., Saunders, 1924, p. 76.

<sup>19</sup> Foot, N. C., and Mènard, M. C. *Arch. Path.*, 4: 211, 1927.

of the cells somewhat but the staining of the mitochondria adds so much to the facility in discrimination of the cells that its disadvantages are counterbalanced. To make a preparation of fresh blood on the dry film of stain, the size of the drop is important and can be learned only by experience. The drop must spread evenly and must be large enough to occupy the space between the slide and the coverslip without any overlapping of the red cells. As soon as the blood has spread, the edges of the coverslip are sealed with vaseline of a melting point sufficiently high that it will not be softened in the warm-box. The vaseline is applied conveniently from a syringe with a cut-off needle. The preparations should be kept at room temperature until they are counted. They will keep at room temperature in excellent condition for at least two hours. The cells are damaged less by cold than by over-heating and Casey and Rosahn<sup>20</sup> have shown that the supravital reaction is retained for twenty-four hours at ice-box temperature. The slides may be counted at room temperature, but in this case the cells show little or no motility. The optimum conditions for counting the cells are to have the microscope placed in a warm-box with the temperature controlled at 37°C. by a regulator. The cells are highly sensitive to excessive heat. Active streaming of the granules in the cytoplasm does not take place below 37°C.; above this temperature the white cells are soon killed and the fragmentation of the red cells is so much increased that this phenomenon can be counted on to call attention to defects in a thermostatic regulator.

**a. Red Blood Cells.** In the supravital films the reticulum of reticulocytes is stained with neutral red, since this dye acts exactly as does brilliant cresyl blue. A count of the reticulocytes is, however, not accurate because the concentration of the dye best adapted for the study of the white cells is not adequate for complete staining of the reticulocytes. This is indicated by the fact that there are almost always more reticulocytes to be seen at the edges of the preparations where the dye becomes more concentrated. The last stage of reticulation in the red cell is indicated by one or two small neutral red bodies. This reaction corresponds probably to the Isaacs granule and is the most sensitive indication of the beginning of regeneration of red cells in the marrow. Nucleated red cells, poikilocytes, macrocytes and microcytes are readily seen as in any preparations of fresh blood. Fragmentation of red cells is obvious in fresh blood, the long processes being in active motion before they break off. As Rous and Robertson<sup>21</sup> have shown, it is a constant phenomenon, taking place in the blood stream. It is increased in certain anemic states, but since, as has been said, an increase in temperature

<sup>20</sup> Casey, A. E., and Rosahn, P. D. *J. Lab. & Clin. Med.*, 17: 1263, 1931-32.

<sup>21</sup> Rous, P., and Robertson, O. H. *J. Exp. Med.*, 25: 651; 665, 1917.

stimulates fragmentation of the red cells to such a degree, this phenomenon must be controlled carefully by the thermometer.

**b. White Blood Cells.** In making differential counts of white blood cells it is not possible to obtain exact correspondence between the supravital technique and fixed films. This is due to the fact that more cells rupture in making the fixed films and therefore it is possible that the counts with the supravital technique are a little closer to the number of cells actually in the blood stream. For all studies of human blood, however, fixed films should be made also, both on account of certain advantages, such as the staining of nuclear material and the basophilia of the cytoplasm, and for a permanent record. To make the comparison between the two methods as close as possible, the whole drop must be used for making fixed films. Thus if coverslips are used, both must be saved and mounted on the same slide. For differential counts with the supravital technique, we prepare two films from two different drops of blood and count 100 cells on each. If they agree closely, their average is taken as the count. If they vary widely, other preparations are taken and from 400 to 1000 cells are counted. The counting of this number of cells is not as laborious as with fixed films.

(1) *Neutrophiles*. The neutrophilic, pseudoeosinophilic or heterophilic, and amphophilic granules of the leucocytes stain characteristically a salmon pink with neutral red. They represent the neutral tints of this dye, or, as in the case of the pseudoeosinophilic granules, a more acid reaction. The granules of this type vary much in size, in refractivity, and in stainability in different animals. For example these granules in the leucocytes of rabbits' blood are larger, more refractive and stain less intensely than in human blood. In all of the different bloods, these granules stain darker, more toward the acid range of the dye in myelocytes than in mature leucocytes. At the stage of senility, the granules of the neutrophiles lose their power to react to neutral red and become highly refractive. The cell then passes into the so-called non-motile phase. It must be stated that the number of cells showing this reaction is greatly increased in preparations that are excessively thin because in them there is undue pressure on the cells from the weight of the coverslip. It has been shown by de Aberle<sup>22</sup> that the discrepancies concerning the proportion of leucocytes to lymphocytes in the blood of rodents can be cleared up by using the supravital technique, because the neutrophilic granulations, so tiny in these animals, are clearly brought out by the neutral red.

Besides the specific granulations, the neutrophilic leucocytes frequently have vacuoles which stain in neutral red and indicate phago-

<sup>22</sup> de Aberle, S. B. *Am. J. Anat.*, 40: 219, 1927-28.

cytosis. These vacuoles always show a different shade of neutral red, a little more toward the acid range of the dye, than the granules and they are also larger. Indeed they may become quite large. In addition to the specific granules, the leucocytes have mitochondria, usually small in size, which can be seen unstained and are brought out by Janus green. In the older leucocytes they become fewer until they finally disappear entirely in the senile forms. All of the granules and vacuoles flow ceaselessly in the streaming of the cytoplasm which accompanies locomotion. In the supravital preparations when viewed at 37°C., the neutrophilic leucocytes are in constant ameboid motion, which serves as an excellent point of discrimination from lymphocytes and monocytes, each of which has a different type of motility.

(2) *Basophiles*. The granules of the basophilic leucocytes stain with the acid or scarlet reaction of neutral red. These granules are not quite uniform in size and a few of them may react more intensely to the dye than others; this is especially true with the basophile of human blood. The basophilic leucocyte of the blood shows active ameboid movements, but the so-called tissue basophile or mast-cell shows little streaming of the cytoplasm and hence is more like a myelocyte. The presence of vacuoles has not been detected.

(3) *Eosinophiles*. The large refractile granules of the eosinophilic leucocytes react characteristically in neutral red, with the alkaline reaction of the dye. In some samples of neutral red they stain a coppery red, but the perfect reaction is a clear yellow. Occasionally, there are a few tiny basophilic granules to be seen in the eosinophiles and this is the rule in this type of cell in guinea pigs. The eosinophiles are not commonly vacuolated. The technique is more sharply differential for eosinophiles than fixed films; as is well known, an occasional leucocyte in fixed films has granules slightly larger than the average neutrophile, giving a cell difficult to discriminate. The question does arise with the supravital technique for the dye as a chemical indicator is sharply differential. The eosinophiles show active ameboid motion but it is not maintained on the slide for as long as in the case of the neutrophilic leucocytes.

(4) *Lymphocytes*. The characteristic features of lymphocytes are the clear cytoplasm, the large content of mitochondria, usually in the form of rods, and the type of motility. Lymphocytes do not start to move as soon after the slide has been prepared as leucocytes; of the three sizes, small, intermediate and large, the intermediate forms are most often found in motion, but McCutcheon<sup>23</sup> has shown that all lymphocytes will eventually move if the preparations are kept in the warm-box long

<sup>23</sup> McCutcheon, M. *Am. J. Physiol.*, 69: 279, 1924.

enough. The type of motility is characteristic; the nucleus stays in the front end of the cell, not dragged passively in the rear, as it were, as in the ameboid motion of the leucocyte; then the lymphocyte is not in constant motion but rather darts forward and then rounds up and rests. The motility of the cell is best seen in motion pictures of cells in hanging drop preparations as developed by Carrel, Ebeling and Rosenberger and by Warren Lewis. The essential characteristics of its motion can, however, be made out in the supravital films. As has been said, the motility is inhibited by the Janus green but this dye is of great importance in the discrimination of lymphocytes from monocytes, in which the mitochondria are smaller. All cells take up the Janus green more slowly than the neutral red and hence about fifteen minutes should be allowed before counting films with the double stain. The neutral red is evident in the cells as soon as the preparation can be focused. Lymphocytes show a few vacuoles which stain with the neutral red; they may also show one or two refractive bodies. The basophilic substance characteristic of all young blood cells appears yellow in the living cell. By this property Wiseman<sup>24</sup> was able to show that the lymphocytes of the blood show phases of maturation similar to those of leucocytes. According to him, the count of the lymphocytes corresponding to the Arneth-Schilling count is best made with fixed films.

(5) *Monocytes*. The supravital technique is of especial advantage for the discrimination of the monocyte. The monocytes have a denser cytoplasm than the lymphocytes and contain bodies, which we judge to be vacuoles and which react to neutral red. The number of these vacuoles can be made to increase by exposing the cell to materials, such as lipoids, which the cells phagocytose. These vacuoles react with a pink shade to the neutral red, a little farther to the acid reaction than the strict neutral reaction of the dye. In the monocytes in the blood of animals these vacuoles are usually arranged in a rosette around the centrosome; in the corresponding cell of human blood they show a greater tendency to be scattered in the cytoplasm. The monocyte of the blood is a young cell, showing less maturation than the leucocyte; hence its cytoplasm is always basophilic and its centrosome obvious. There are abundant tiny mitochondria, smaller than those of lymphocytes, arranged in the edge of the rosette of vacuoles. The surface film of the monocyte is of great importance, since it is the organ of its function, namely phagocytosis. This surface film is to be seen to best advantage in moving pictures, in which it appears in constant and beautiful veil-like motion around the entire sphere of the cell. In the space between slide and coverslip these films are much hampered in their motion and the

<sup>24</sup> Wiseman, B. K. *J. Exp. Med.*, 54: 271, 1931; *Folia haematol.*, 46: 346, 1931-32.

cell may appear spread out in the form of a triangle, with the nucleus a little excentrically placed and the surface film in folds around the border. With the supravital technique, variations in the functional state of this cell are readily made out. These consist either of a great increase in large vacuoles, the type which is seen in Malta fever,<sup>25</sup> or of a marked increase in tiny vacuoles.<sup>26</sup> This process gives all the transitions between the monocyte and the epithelioid cell as seen in tuberculosis and other pathological conditions.

c. **Cells of Leucemic Blood.** For blood in which there are large numbers of immature cells, as in leucemic blood, the combination of the neutral red and Janus green is the preferable stain. Myeloblasts<sup>27</sup> are discriminated by large numbers of small mitochondria scattered diffusely throughout a deeply basophilic cytoplasm, which appears yellow in the living cell. Since myeloblasts in the blood occur practically always in association with the earliest type of myelocyte, namely type-A, an oxidase preparation should always be made for this discrimination. The myelocyte type-A has a few specific granules and in both neutrophilic and eosinophilic myelocytes, this first clump of granules, readily seen in the supravital technique, gives one positive granule in the oxidase reaction. For this technique, the method of Sato and Sekiya<sup>28</sup> is the most practical since their solutions are stable. The myelocytes do not show streaming of the granules in the supravital technique and no ameboid motion. The streaming of the cytoplasm first becomes marked at the stage of the metamyelocyte. The most difficult discrimination to be made in films of blood is between the monocyte and the B-myelocyte when it has about half of the full quota of granules. This is true both in fixed and in supravital films. Indeed for this discrimination, the surface film and the type of motility to be seen in the living state are the most important factors so far known. In human blood both types are oxidase-positive.

In leucemic blood, it frequently happens that many of the cells are so fragile that they fracture in making the fixed films. In certain cases of lymphatic leucemia all of the cells make the so-called smudges in fixed films. In these cases the supravital technique is the only one to be used,

<sup>25</sup> Sabin, F. R. *Bull. Johns Hopkins Hosp.*, 34: 277, 1923.

<sup>26</sup> Sabin, F. R., Smithburn, K. C., and Thomas, R. M. *J. Exp. Med.*, 62: 751, 1935.

<sup>27</sup> Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A. *J. Exp. Med.*, 40: 845, 1924.

Simpson, M. E., and Deming, J. M. *Anat. Rec.*, 27: 218, 1924. *Folia haematol.*, Teil 1, 34: 103, 1927.

<sup>28</sup> The Sato and Sekiya technique is given by Sato, A., and Yoshimatsu, Sh. *Am. J. Dis. Child.*, 29: 301, 1925.



but in general fixed films should be made, studied and kept as permanent records of all abnormal bloods.

### 2. Cells of the Bone Marrow

For the differential counts of the cells of bone marrow, now an important part of the study of bone marrow removed at biopsy, the supravital technique is the method of choice. The double stain should be used. If the marrow is relatively fluid, as in very young animals, a tiny drop of the cells can be drawn a short distance into a pipette, transferred to a slide and spread by the mere weight of the coverslip. When fat is present, a tiny bit may be cut out, covered and spread with very gentle and steady pressure on the coverslip. All spicules of bone must be avoided. The preparation is then sealed as already indicated. In the areas in which the cells are well stained and lie one cell deep, differential counts may be made. Since the youngest white cells, the myeloblasts, have a yellow cytoplasm due to the basophilia, differences between the nuclear patterns of erythroid and myeloid cells are important in the discrimination. Since the erythroid and myeloid cells grow in islands in the marrow, a larger number of cells should be included in the counts than for blood cells. In experimental work we make 5000 cells the standard; for diagnostic work 1000 will suffice.<sup>29</sup>

### 3. Cells in Fluids Other Than Blood Plasma

In general cells tend to deteriorate more quickly in other body fluids, such as cerebrospinal fluid, than in blood plasma. Kubie and Shults<sup>30</sup> have shown that the supravital technique allows a sharper discrimination of the free cells of *cerebrospinal fluid* than other methods. Material from exudates of the body cavities, from joints and lymph nodes can be obtained through pipettes and mounted as blood films. Cells can be obtained from the peripheral sinuses of lymph nodes by introducing a fine pipette tangentially to the gland; the supravital technique shows that this fluid is more toxic to cells than the fluid from the central sinuses or from the thoracic duct.

### 4. Cells of the Connective Tissues

The omentum, together with the cells of peritoneal exudates, and the cells of the retrosternal lymph nodes, which drain the peritoneal cavity, make the best material for the study of the reactions of the cells

<sup>29</sup> Sabin, F. R., Miller, F. R., Smithburn, K. C., Thomas, R. M., and Hummel, L. E. *J. Exp. Med.*, 64: 97, 1936.

<sup>30</sup> Kubie, L. S., and Shults, G. M. *J. Exp. Med.*, 42: 565, 1925.

of the connective tissues to various stimuli. This is true because the omentum can be split into its two folds and mounted on a slide with a dry film of stain. Some accessory fluid must be added between omentum and coverslip and neutral red in saline is used for this purpose. The preparations need not be sealed with vaseline if a large enough coverslip is used; this makes it simpler to fix the preparation after it has been studied supravitaly. If the coverslip is left in place for about one hour the film of omentum will stick to the slide and can then be handled as a section. The advantage of these films of the omentum is that the cells can be seen in them in their natural relationship to each other. For comparison with the omentum, scrapings of the omentum, of the peritoneal wall, of the diaphragm, as well as from the sinuses of the lymph nodes already mentioned, may be studied with the supravital method. These preparations need no accessory fluid.

Supravital preparations of the subcutaneous tissues are best made by inducing a slight edema by injecting the neutral red in saline into the tissues. One half of an area to be studied can be fixed for sections and the other half made edematous for the study of the living cells. The supravital preparations are made by gentle scraping of a freshly cut surface and mounting as for blood films.

**a. Monocytes and Macrophages (Clasmatocytes).** These two types of cells, making together the phagocytic mononuclear forms, are closely allied. They have exactly the same type of surface films, the organ of their function, and they move in precisely the same manner. The monocytes of the tissue, few in number, unless artificially stimulated to increase, have the deeply basophilic cytoplasm of young cells. With the supravital technique it is possible to follow to some extent the degree of change which is brought about by these cells upon phagocytosed material. Two types of activity may be discriminated. For example some materials, such as a phagocytosed leucocyte, appear to be dissolved within the vacuole which first forms around them. This process takes place rapidly, involving from twenty-four to forty-eight hours only. When several leucocytes have been engulfed, the different vacuoles in the same cell can be seen to be at different pH values with neutral red as indicator, that is, some will be a bright red while others are yellow. This process suggests an enzyme action.<sup>31</sup> Other materials, notably lipoids, are not quickly dissolved by cells; rather they are dispersed into finer and finer particles and acted upon slowly by cells. By this type of

<sup>31</sup> Sabin, F. R., Doan, C. A., and Forkner, C. E. *J. Exp. Med., Sup.* No. 3, 52: 1, 1930, see p. 79.

reaction a monocyte may become an epithelioid cell.<sup>32</sup> These facts suggest that the terms monocyte and macrophage or clasmatocyte may refer to different kinds of functional activities within a single strain of cells.

**b. Fibroblasts.** Fibroblasts are discriminated from the phagocytic mononuclears by showing less reaction to the neutral red and by growing more as a tissue, with each cell having a relation to those around it, rather than as free wandering cells.

**c. Primitive Connective Tissue Cells.** These cells, whether in the areolar tissues or in lymph nodes or bone marrow, show less reaction to the supravital dyes than the other cells of the connective tissues and they have less chromatin in their nuclei.

**d. Plasma Cells.** Plasma cells<sup>33</sup> are very distinctive in the supravital reaction. They have the most dense cytoplasm of any of the connective tissue cells; it is deeply basophilic and appears a yellowish grey in the living state. There are very many tiny mitochondria diffusely scattered; the nuclei are characteristic and the clear zone beside the nucleus is evident. After they have been once identified they are not readily confused with any other type. Their final stage, the cells with the Russell bodies, is also distinctive.

### 5. Cells From Organs

In the study of the different organs both the parenchyma of the organs and their connective tissue cells are to be considered. The supravital technique has been applied most extensively to such organs as *lymph nodes, spleen* and *bone marrow* in which the mesenchymal cells represent the functional cells. The preparations of these organs can be made either by puncture with pipettes or by scraping the cut surface.

Scrapings from the lungs show when there is an increase in the free phagocytic cells over the normal condition. The epithelium of the bronchi comes off in sheets, readily discriminated by the cilia. The Kupffer cells of the liver can be studied in scrapings from a cut surface, but many of them are so fragile that they are damaged or ruptured with this method. They are obtained to much greater advantage by the ingenious method of Rous and Beard,<sup>34</sup> who induced them to phagocytose particles of iron and then washed them out of the vessels by perfusion. Too little is known about the variations in appearance of liver cells due to varying functional states at the present time, to discriminate any but the most extreme abnormal states.

<sup>32</sup> Sabin, F. R., Smithburn, K. C., and Thomas, R. M. *J. Exp. Med.*, 62: 751: 771, 1935.

<sup>33</sup> Miller, F. R. *J. Exp. Med.*, 54: 333, 1931.

<sup>34</sup> Rous, P., and Beard, J. W. *J. Exp. Med.*, 59: 577, 1934.

Beard, J. W., and Rous, P. *J. Exp. Med.*, 59: 593, 1934.

Only in the case of brain tumors, through the extensive studies of Eisenhardt and Cushing,<sup>35</sup> and in the cellular reactions in tuberculosis<sup>36</sup> and syphilis,<sup>37</sup> have thorough applications of the technique been made to pathological conditions.

<sup>35</sup> Eisenhardt, L., and Cushing, H. *Am. J. Path.*, 6: 541, 1930.

<sup>36</sup> Sabin, F. R., Doan, C. A., and Forkner, C. E. *J. Exp. Med.*, Sup. No. 3, 52: 1, 1930.

<sup>37</sup> Cunningham, R. S., Morgan, H. J., Tompkins, E. H., and Harris, S. *Am. J. Syphilis*, 17: 515, 1933.

Morgan, H. J., Harris, S., Tompkins, E. H., and Cunningham, R. S. *Am. J. Syphilis*, 17: 522, 1933.

## CHAPTER III

### BACTERIOLOGICAL METHODS

H. J. CONN, F. B. MALLORY AND FREDERIC PARKER, JR.

GENERAL CONSIDERATIONS 132. STUDY OF PURE CULTURES 133. Unstained preparations 133. Stained preparations 134. STUDY OF BACTERIA IN NATURAL HABITAT 146. In soil 146. In dairy products 147. SECTIONED MATERIAL 148. Gram-negative bacteria 149. Gram-positive bacteria 152. Acid-fast bacteria 153.

#### A. GENERAL CONSIDERATIONS

The preparation of specimens for microscopic examination is simpler in the case of bacteria than in that of higher plants or animals, except when some special technique such as flagella staining is in question. It is, however, very important that a correct optical system be used; and the microscope should be given special consideration.

The microscopes most commonly used in bacteriological laboratories are equipped with Abbé condenser, triple nosepiece bearing 1.9 mm., 8 mm. (or 4 mm.) and 16 mm. objectives, with oculars of 6.4 $\times$  and 10 $\times$  magnifications. Little attention need be given to the selection of the lower-powered objectives; but the 1.9 mm. oil-immersion objective and the oculars should be carefully selected. The oil-immersion lens for best results should be apochromatic; but on account of expense and the care necessary in handling such an objective, the apochromatic type is ordinarily less practical than the fluorite objective of 1.9 mm. The latter, although having a lower numerical aperture than the apochromatic lenses, gives sufficient definition so that it may be used with quite high-powered oculars, and is entirely satisfactory for ordinary bacteriological work. If a fluorite objective is employed, the oculars for routine use may be 7.5 $\times$  and 12.5 $\times$ , instead of the 6.4 $\times$  and 10 $\times$ , because of the greater definition secured; and in this way greater magnification may be obtained. The oculars of 10 $\times$  and below may be of the Huygenian type; but for best results the higher-powered oculars should be the compensating type. An ocular as high as 15 $\times$  compensating may be used with a 1.9 mm. fluorite objective; and in the study of minute bacteria such a combination is often very useful. For routine work, however, the 6.4 $\times$  and 7.5 $\times$  oculars are most satisfactory.

## B. STUDY OF PURE CULTURES

### I. Unstained Preparations

1. **Hanging Drop.** Unstained preparations of bacteria are generally utilized for determining whether a culture is motile. The important points to be observed in making them, therefore, are that the culture be at its greatest vigor, and that the preparation be so made as not to hinder the motility of the organisms. Hanging-drop preparations are usually employed.

Cultures for this purpose should be inoculated into whatever medium, either liquid or solid, is understood to be most favorable for their growth. They should be incubated at optimum temperature until good growth has taken place, ordinarily about twenty-four hours. In the case of liquid cultures, a loopful should be placed on the coverslip; in the case of solid cultures (agar), the same method may be used if sufficient liquid has been exuded at the base of the tube, or if not, a minute portion of the surface growth may be mixed with a small drop of water on the coverslip. The coverslip bearing a drop of culture should be inverted over the hollow of a depression slip, with care to see that the drop does not touch the glass at any point. If a depression slip is not available, good results may be obtained by a slightly different technique which does not give true hanging-drop conditions. A cover glass is supported by two or three small pieces of a broken coverslip placed on an ordinary microscopical slip. If this latter technique is used, one does not need to take precautions to prevent the drop touching the glass; for motility can take place between slip and cover almost as freely as in a hanging drop.

The preparation should be examined with a dry objective of about 4 mm. focus. If the bacteria are very small, a comparatively high-powered ocular (as for example 12.5 $\times$ ) may be used to give the desired magnification; but for ordinary purposes, an ocular of 6.4 $\times$  or 7.5 $\times$  is more satisfactory. Artificial light must be used, either with or without a "day-light" screen, and the diaphragm below the condenser should be nearly closed.

2. **Study of Microscopic Colonies.** There are three ways of making a microscopical study of unstained bacterial colonies: (1) direct examination on the culture plate; (2) transfer of the colony to a microscopical slip; (3) agar block or gelatin drop culture.

(1) Direct examination on a culture plate requires a rather low power and does not give very satisfactory results. It is ordinarily employed

only when precautions must be taken to avoid contamination of the culture plate.

(2) Removal of the bacterial colony is accomplished by cutting out a block of the medium and lifting it up with scalpel or spatula. For this purpose the colony should preferably be growing in agar; removal of a gelatin colony for microscopical examination is a much more difficult procedure. The block of medium containing the colony should be placed on a slip with the colony to be studied at or near the top of the block. It may then be covered with a coverslip and examined with a relatively high power. An oil-immersion objective may sometimes be used to good advantage; but ordinarily the same combination of lenses should be used as mentioned in the case of hanging drop.

(3) The agar block or gelatin drop culture is a little more difficult to study. Slips with deep depressions must be used for this work and should be sterilized before using, together with coverslips to fit them. They may be sterilized inside of Petri dishes and kept inside of them until used. For an agar block culture, a small piece of sterile agar should be cut out of a Petri plate in which it has been poured without inoculation. It should be cut and removed with a previously flamed scalpel or spatula, and transferred under aseptic conditions to one of the sterile coverslips. Just before placing on the coverslip, the culture to be studied should be inoculated on the surface at one minute spot, and the inoculated side should be placed against the coverslip. The coverslip bearing the block should then be inverted into the depression of the slip and should be sealed in such a way as to prevent evaporation. For sealing the coverslip in place, pyroxylin cement gives very good results.

The gelatin drop culture is managed a little differently. Ordinarily the inoculation is effected by placing a minute amount of bacterial growth on the sterile coverslip. A drop of sterile gelatin which has been melted and then cooled down almost to its solidifying point is placed on the spot thus inoculated. The drop of inoculated gelatin is covered with the depression slip and immediately transferred to a refrigerator or other cooling device until the gelatin solidifies; it is then sealed around the edges of the coverslip.

These agar block or gelatin drop cultures, after incubation (not higher than 20°C. in the case of gelatin), are to be examined by the same methods as just described for other unstained preparations.

## II. Stained Preparations

1. **Preparations for Staining.** For a few purposes bacteria are stained without drying by adding dilute stain to the liquid in which they are suspended. They are then examined without washing, or else

the washing is done beneath the coverslip by introducing water from one side and blotting the stain out from the other. Ordinarily, however, bacteria are stained in dried smear preparations.

Smear preparations are simply made by mixing a small amount of the bacterial growth (best taken from the surface of agar) with a drop of water (preferably distilled) on a slip. Care should be taken not to use enough of the bacterial growth to give noticeable turbidity. The drop is then spread over an area of one or two square centimeters, according to the amount of liquid in the drop, and is allowed to dry. No fixative is needed, because the bacterial growth ordinarily is sufficiently gelatinous in its nature to stick to the glass without the addition of any fixing agent. Mild heat is sometimes used so that bacteria will adhere more firmly, but it is not ordinarily necessary. The smears should never be heated above the boiling temperature.

Occasionally difficulty in obtaining a good smear preparation is experienced because of the presence of slimy substances produced by the bacteria. Smear preparations from such organisms cannot well be stained in the usual way, either because the entire mucous mass takes this stain and obscures the bacteria or else because nothing in the preparation stains. Such organisms are sometimes handled by centrifuging to remove the bacteria from the slime; the bacteria at the bottom of the centrifuge tube are then washed with water and smeared as usual on the slip. In the writer's experience, it has proved much simpler to make preparations from such organisms in the usual manner and then to stain with some special dye such as rose bengal (p. 146).

**2. Staining Methods.** The most recent summary for staining procedures for pure culture study of bacteria is part of the "Manual of Methods for Pure Culture Study of Bacteria," published by the Society of American Bacteriologists (Geneva, N. Y.) and issued by the Committee on Bacteriological Technic. This summary of staining methods is given in Leaflet iv of the Manual entitled "Staining Procedures," of which the fifth edition was issued in May, 1934. Permission has been secured from the committee to quote these staining procedures here. Attention, however, is called to the fact that the Manual of Methods is a loose-leaf publication constantly subject to revision, and that the committee assumes no responsibility for the methods being up-to-date for more than a brief period after the date of issue of each section of the publication. To obtain the most recently approved methods, therefore, the reader is referred to the publication in question which the committee plans to keep up-to-date.<sup>1</sup>

<sup>1</sup> Committee on Bacteriological Technic. Manual of Methods for Pure Culture Study of Bacteria. Publ. by Soc. of Amer. Bacteriologists, Geneva, N. Y., 1923, with revisions of various sections at later dates. A loose-leaf publication.



The following nine pages are quoted from this leaflet:

In the literature concerning staining methods there is a surprising amount of inaccuracy. Few of the formulae found can be accepted in the light of present knowledge without some degree of interpretation to make them more explicit; in some cases the author's intentions in the matter are evident, in others they can be only inferred. Hence, in the following pages, two formulae for some of the staining solutions are given: the original one (or that generally found in text books); and the emended formula as interpreted by the committee. The latter is intended to give the same solution as probably employed in the first place; but the committee assumes no responsibility for the identity of the two and offers the emendation merely to prevent the perpetuation of formulae which are clearly ambiguous or indefinite as to their ingredients.

In using any of the methods it must be remembered that a blind adherence to a staining technique is no guarantee that the result will be satisfactory. Even experienced workers sometimes discover to their dismay that they took too much for granted as to the purity of their reagents, cleanliness of slides<sup>2</sup> and covers, or proper compounding of the staining solutions. A technique should, therefore, be checked upon known organisms as controls. It is, furthermore, important to know that the solutions and water used for dilution are reasonably free from bacteria and their spores.

#### A. General Bacterial Stains.<sup>3</sup>

##### (1) Ziehl's Carbol-fuchsin

Old Statement of Formula	Emended Statement of Formula <sup>4</sup>
	Solution A
Sat. Alc. Sol. basic fuchsin . . . . . 10 c.c.	Basic fuchsin (90 per cent
5 per cent sol. carbolic acid . . . . . 100 c.c.	dye content) <sup>5</sup> . . . . . 0.3 gm.
	Ethyl alcohol (95 per cent) . . . . . 10 c.c.
	Solution B
	Phenol . . . . . 5 gm.
	Distilled water . . . . . 95 c.c.
	Mix Solutions A and B.

<sup>2</sup> "Slide" in this quotation is equivalent, in most cases, to the term "slip," used elsewhere in this book.

<sup>3</sup> Loeffler, F. *Mitt. a. d. k. Gesundheitsamte*, 2: 421, 1884.

Ziehl, F. *Deutsch. Med. Wchnschr.*, 8: 451, 1882.

<sup>4</sup> The emended statements of the formulae in the following pages are not formulae especially recommended by the committee; but represent an attempt to put the original formulae in more up-to-date form and to show what the original author probably intended. The committee assumes no responsibility for the actual correctness of its interpretation. Corrections and comments from users are invited.

<sup>5</sup> It is not necessary that dry stains of the exact dye content specified be used in this or in the following formulae. Samples of higher or lower dye content may be employed by making the proper adjustment in the quantity used.

(2) *Loeffler's Alkaline Methylene Blue*

Original Statement of Formula	Emended Statement
	Solution A
Conc. Sol. methylene blue in alcohol..... 30 c.c.	Methylene blue (90 per cent dye content)..... 0.3 gm.
Sol. KOH in distilled water (1:10,000)..... 100 c.c.	Ethyl alcohol (95 per cent) 30 c.c.
	Solution B
	Dilute KOH (0.01 per cent by weight)..... 100 c.c.
	Mix Solutions A and B

This formula is still very useful, although no longer as necessary as formerly. The use of a weakly alkaline solution was apparently to overcome the acidity of poorly neutralized dyes formerly on the market and to secure partial polychroming of the methylene blue. The methylene blue at present available, however, especially that which is certified by the Commission on Standardization of Biological Stains, does not require neutralization, and contains a sufficient admixture of the lower homologues of methylene blue (azure, etc.) to give good differentiation without treatment with alkali. As a result, good results can ordinarily be obtained if distilled water is substituted for the dilute alkali in the above formula.

(3) *Aniline Gentian Violet (Ehrlich)\**

Original Statement of Formula	Emended Statement
	Solution A
Sat. Alc. Sol. Gentian violet 5-20 c.c.	Crystal violet (85 per cent dye content) ..... 2.5 gm.
Aniline water (2 c.c. aniline shaken with 98 c.c. water and filtered) ... 100 c.c.	Ethyl alcohol (95 per cent) .. 12 c.c.
	Solution B
	Aniline ..... 2 c.c.
	Distilled water ..... 98 c.c.
	Shake and allow to stand for a few minutes, then filter.
	Mix Solutions A and B

The use of crystal violet in this formula is recommended because this dye is more uniform than the various products sold as gentian violet.

\* Although various aniline water formulae for this dye are known as Ehrlich's, he seems properly to be credited only with the idea of using aniline water in the formula. Various subsequent authors have modified the solution to suit themselves; and as a result the amount of gentian violet recommended in different places varies to the extent shown in the left-hand column above.

The committee, however, does not recommend the use of an aniline formula in the Gram stain, but rather one of the two given below.

*B. The Gram Stain.* There are a large variety of modifications of the Gram stain, many of which have recently been listed by Hucker and Conn. Two of the more satisfactory of the recent modifications may be mentioned.

(1) *Hucker Modification (recommended by the Committee)*

Ammonium Oxalate Crystal Violet

Solution A

Crystal violet (85 per cent dye content) .....	4 gm.
Ethyl alcohol (95 per cent) .....	20 c.c.

Solution B

Ammonium oxalate .....	0.8 gm.
Distilled water .....	80 c.c.

Mix Solutions A and B

Lugol's Iodine Solution

Iodine .....	1 gm.
Potassium iodide .....	2 gm.
Distilled water .....	300 c.c.

Counterstain

Safranin (sat. solution in 95 per cent alcohol) .....	10 c.c.
Distilled water .....	100 c.c.

*Technique.* Stain one minute with the crystal violet solution; wash in water; immerse in iodine for one minute; wash in water and blot dry; decolorize in 95 per cent alcohol for thirty seconds with gentle agitation; blot and cover with counterstain for ten seconds; then wash dry, and examine with oil-immersion lens.

(2) *Kopeloff and Beerman Modification (recommended by the Committee)*

Alkaline Gentian Violet

Solution A

Gentian or crystal violet <sup>7</sup> .....	1 gm.
Distilled water .....	100 c.c.

<sup>7</sup> The authors specify either crystal violet or methyl violet 6B. Probably any of the gentian violets now sold under the Commission certification would be satisfactory; they are ordinarily either crystal violet or one of the bluer grades of methyl violet.

## Solution B

Sodium bicarbonate .....	1 gm.
Distilled water .....	20 C.C.

Just before use, mix 1.5 c.c. of solution A with 0.4 c.c. of solution B in a beaker.

## Iodine Solution

Iodine .....	2 gm.
Normal solution sodium hydroxide.....	10 C.C.

After the iodine is dissolved make up to 100 c.c. with water.

## Counterstain

Basic fuchsin .....	0.1 gm.
Distilled water .....	100 C.C.

Technique. Stain five minutes or more with the alkaline gentian violet solution; rinse with the iodine solution; add fresh iodine solution and allow to stand two minutes or longer; drain off iodine solution and blot dry (without washing); decolorize with 100 per cent acetone, adding drop by drop to the slip while tilted until no color is seen in drippings (generally less than 10 seconds); dry in the air; counterstain for ten to thirty seconds; wash in water, dry, and examine with oil-immersion lens.<sup>8</sup>

*C. Acid-fast Staining.* Various methods have been given for determining the acid-fast properties of an organism; but all are really variations of the same general procedure: staining deeply with carbol fuchsin, then decolorizing with acidified alcohol, followed or accompanied by a counterstain.

(1) *The Ziehl-Neelsen method*<sup>9</sup> is: carbol fuchsin with gentle steaming for three to five minutes or cold for fifteen minutes; wash in water; decolorize in 95 per cent ethyl alcohol containing 3 per cent by volume concentrated hydrochloric acid until only a suggestion of pink remains; wash in water; counterstain with saturated aqueous methylene blue or Loeffler's methylene blue; wash and dry.<sup>10</sup>

(2) The Ziehl-Gabbet method is similar but calls for simultaneous decolorizing and counterstaining in 2 per cent methylene blue in 25 per cent sulphuric acid (sp. gr. 1.18).<sup>11</sup>

(3) Much's method No. 2, which is now quite widely used, employs carbol

<sup>8</sup> Hucker, G. J. *Abstr. Bact.*, 6: 2, 1922.

Hucker, G. J., and Conn, H. J. *N. Y. Agr. Exp. Sta., Tech. Bull.* 93, 1923.

Kopeloff, N., and Beerman, P. *J. Inf. Dis.*, 31: 480, 1922.

<sup>9</sup> See Stitt's *Practical Bacteriology, Blood Work, and Animal Parasitology*, Ed. 7, Phila., 1923, p. 58.

<sup>10</sup> Compare with method given in Mallory's section on Acid-fast Staining of Sections (p. 153).

<sup>11</sup> Gabbet, H. S. Rapid staining of the tubercle organism. *Lancet*, 1: 757, 1887.

gentian violet of essentially the formula already given except that in the place of crystal violet the author calls for methyl violet BN. Preparations are stained cold for twenty-four hours or by gentle application of heat until steaming. They are then washed in water and treated with Lugol's iodine solution from one to five minutes. After a second washing they are treated with 5 per cent nitric acid for one minute followed by 3 per cent hydrochloric acid for ten seconds. They are then decolorized one minute in equal parts of acetone and 95 per cent ethyl alcohol. Weiss (1909) has modified this procedure by staining with a mixture of 3 parts of carbol fuchsin to 1 part of carbol gentian violet and counterstaining with one per cent aqueous safranin (five to ten seconds) or with Bismarck brown (one minute). The counterstain is applied immediately after the decolorization, the acetone-alcohol being removed merely by blotting. In some laboratories this method of counterstaining is employed following the Much technique with carbol gentian violet alone for the primary stain.<sup>12</sup>

(4) The Cooper method calls for staining in Ziehl's carbol fuchsin to which 3 per cent of a 10 per cent aqueous sodium chloride solution is added just before use. Smears are stained either by steaming three to four minutes, then allowing them to cool until a precipitate forms, or else by standing over night in a 37° incubator and cooling in an ice box for twenty minutes to allow precipitation to occur. After the precipitation has taken place, wash with tap water and decolorize one to ten minutes in acid alcohol (5 c.c. of nitric acid, sp. gr. 1.42, to 95 c.c. of 95 per cent ethyl alcohol). Wash again with water, and finally for one minute with 95 per cent ethyl alcohol. Counterstain with 1 per cent brilliant green, or if the smear is heavy, with a greater dilution of this same stain. Wash with water, dry, and examine.<sup>13</sup>

*D. Spore Stains.* The older methods of spore staining are very similar to those for acid-fast staining, differing largely in the manner of applying heat during the staining and in that the acid used in decolorizing is diluted with water instead of with alcohol. Recent methods are somewhat different:

(1) *Dorner's Method (recommended by the Committee)*<sup>14</sup>

(a) *For bacteria not forming slime:*

- (1) Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test-tube.
- (2) Add an equal quantity of freshly filtered Ziehl's carbol fuchsin.

<sup>12</sup> Much, H. Über die granuläre, nach Teil nicht färbbare Form des Tuberkulosevirus. *Beitr. z. Klin. d. Tubercul.*, 8: 85-99, 1907.

Weiss, L. Zur Morphologie des Tuberkulosevirus unter besonderer Berücksichtigung einer Doppelfärbung. *Berl. klin. Wchnschr.*, 46: 1797-1800, 1909.

<sup>13</sup> Cooper, F. B. A modification of the Ziehl-Neelsen staining method for tubercle bacilli. *Arch. Path. & Lab. Med.*, 2: 382-5, 1926.

<sup>14</sup> Dorner, W. *Jahrb. d. Schweiz.*, 36: 595, 1922.

Dorner, W. *Le Lait*, 6: 8, 1926.

- (3) Allow the mixture to stand in a boiling water bath for ten to twelve minutes.
- (4) On a coverslip or slide mix one loopful of the stained preparation with one loopful of a saturated aqueous solution of nigrosin.
- (5) Smear as thinly as possible and do not dry too slowly.

(2) *Recent Modifications of the Wirtz Method*<sup>15</sup>

(a) *Schaeffer and Fulton Modification (recommended by the Committee)*

Bacterial smears are made as usual and fixed in a flame. They are flooded with 5 per cent aqueous malachite green for thirty to sixty seconds, and heated to steaming three or four times. The excess stain is washed off in running water for about half a minute, and 0.5 per cent aqueous safranin is added for about thirty seconds. The smears are then washed and blotted. The spores should be stained green, the rest of the cells red.<sup>16</sup>

(b) *Conklin Modification*

Smears are prepared as above, and are flooded with 5 per cent aqueous malachite green. They are steamed for ten minutes, keeping the slide flooded by addition of fresh staining fluid. They are then washed one-half minute in running water, and counterstained with 5 per cent aqueous mercurochrome for one minute. The picture is the same as with the Schaeffer and Fulton technique.<sup>17</sup>

In both of these last two procedures trouble is sometimes experienced with the green fading after the slides have stood a few days. Apparently this is due to an alkaline reaction and can be prevented by treating the slides in acid before making the smears. ("Clean" slides are sometimes covered with an invisible film of soap or washing powder which can interfere with this stain unless neutralized.)

*E. Flagella Stains.* Flagella staining is an extremely difficult technique and there have been more different methods proposed for the purpose than for any other bacteriological procedure. There is no evidence that any one of these procedures is universally better than any other. The important thing is that one method be selected and that the user become so familiar with it that he can obtain good results; although it is probable that some kinds of bacteria stain better by one method, others by some other. Some of the favorite formulae are as follows:

<sup>15</sup> Wirtz, R. En einfache Art der Sporenfärbung. *Centbl. f. Bakt.*, I Abt., Orig., 46: 727, 1908.

<sup>16</sup> Schaeffer, Alice B., and Fulton, McD. A simplified method of staining endospores. *Science*, 77: 194, 1933.

<sup>17</sup> Conklin, Marie E. Mercurochrome as a bacteriological stain. *J. Bact.*, 27: 30, 1934.

(1) *Loeffler's Flagella Stain*<sup>18</sup>

## (1) Mordant:

Solution of tannic acid (20 per cent in water).....	10 c.c.
Sat. <sup>19</sup> aqu. solution of ferrous sulphate.....	5 c.c.
Sat. solution of basic fuchsin in 95 per cent ethyl alcohol (i.e. about 3 to 5 per cent).....	1 c.c.

## (2) Stain:

Carbol fuchsin.

There are various modifications of this technique. Duckwall,<sup>20</sup> for example, has modified it by using 15 c.c. of a 13.3 per cent solution of tannic acid. The mordant is ordinarily used fresh, and filtered each time before using; although Kulp<sup>21</sup> obtains better results by letting it stand over night and centrifuging before use. Kulp prefers gentian violet to basic fuchsin both in the mordant and in the stain.

Shunk's<sup>22</sup> modification differs so radically from the original that it must be treated separately. It is as follows:

(2) *Shunk's Flagella Stain*

## (1) Mordant:

## Solution A

Sat. aqu. solution of tannic acid.....	30 c.c.
Solution of ferric chloride (5 per cent in water).....	10 c.c.

## Solution B

Aniline .....	1 c.c.
Ethyl alcohol (95 per cent).....	4 c.c.

Solution A is best prepared a week or more ahead of time and filtered before use. When using, place about 9 drops of solution A on the slide and immediately add one drop of solution B; and there is then a precipitation on the slide. The excess mordant is carefully drained off, and the stain applied without previous washing.

## (2) Stain:

Carbol-fuchsin, 1 per cent safranine in 50 per cent alcohol, aniline gentian violet, or Loeffler's methylene blue may be used. Shunk recommends the following, however:

Loeffler's methylene blue solution.....	30 c.c.
Solution B of Shunk's mordant.....	3 c.c.

This solution is immediately ready for use and keeps well.

<sup>18</sup> Loeffler, F. *Centbl. f. Bakt.*, 7: 625, 1890.

<sup>19</sup> By "Sat. solution" (unless otherwise specified) is meant a solution saturated at room temperature (21° to 25°C.).

<sup>20</sup> Duckwall, E. W. *Canning and Preserving of Food Products with Bacteriological Technic*, Pittsburgh, 1905, 458 pp.

<sup>21</sup> Kulp, W. L. *Stain Technology*, 1: 60, 1926.

<sup>22</sup> Shunk, I. V. *J. Bact.*, 5: 181, 1920.

(3) *Casares-Gil's Flagella Stain*<sup>23</sup>

As Published by Plimmer and Paine (p. 144)

## (1) Mordant:

Tannic acid .....	10 gm.
Aluminum chloride (hydrated) .....	18 gm.
Zinc Chloride .....	10 gm.
Basic fuchsin <sup>24</sup> .....	1.5 gm.
Alcohol (60 per cent) .....	40 c.c.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 c.c. of the alcohol first, and then the rest slowly. This alcoholic solution may be kept several years. For use, dilute with 4 parts of water, filter off precipitate and collect filtrate on the side allowing it to act for sixty seconds.

N. B. It has recently been stated (Thatcher<sup>25</sup>) that better results can be obtained in this technique if the mordant is diluted only 1 to 1 instead of 1 to 4. Subsequent investigation by the committee (with the corroboration of Miss Thatcher) shows a dilution of 1 to 2 to be rather more satisfactory.

## (2) Stain: Carbol fuchsin.

(4) *Gray's Flagella Stain*<sup>26</sup>

## (1) Mordant:

## Solution A

Potassium alum. (sat. aqu. solution) .....	5 c.c.
Tannic acid (20 per cent aqu. solution) .....	2 c.c.

(A few drops of chloroform must be added to this if a large quantity is made up.)

Mercuric chloride (sat. aqu. solution) .....	2 c.c.
----------------------------------------------	--------

## Solution B

Basic fuchsin (sat. alc. solution) .....	0.4 c.c.
------------------------------------------	----------

Mix Solutions A and B less than twenty-four hours before using. Both solutions separately may be kept indefinitely, but deteriorate rapidly after mixing.

## (2) Stain: Carbol fuchsin.

The general method of applying flagella stains requires first the procuring of very young and vigorous cultures. The exact method of preparing these cultures varies with different organisms; but, in general, growth from twelve to twenty-four hours old on surface of agar gives best

<sup>23</sup> See Galli-Valerio, B. *Centbl. f. Bakt., I Abt. Orig.*, 76: 233, 1915.

<sup>24</sup> The authors specify rosaniline hydrochloride. There are, however, other basic fuchsins more universally available which ought to prove equally satisfactory.

<sup>25</sup> Thatcher, L. M. *Stain Technology*, 1: 143, 1926.

<sup>26</sup> Gray, P. H. H. *J. Bact.*, 12: 273, 1926.



results. If old stock cultures are used, it is often necessary to make daily transfers for a few days before studying, in order to restore their vigor. Kulp<sup>21</sup> (p. 142) obtains especially good results by using agar slants with plenty of condensation water; then from a twenty-four hour culture a loopful of the condensation water is transferred to a second agar slant, from which, twenty-four hours later, 1 or 2 drops of condensation water are poured off aseptically into a tube of sterile distilled water which has been kept at the same temperature as the culture; the tube is then incubated at the optimum temperature of the organism for from forty-eight to seventy-two hours. A similar procedure was recommended some time ago by Johnston and Mack.<sup>27</sup> Others, however, seem to find that bacteria shed their flagella if left so long in distilled water.

A very important point is that the cover glasses or slides used be scrupulously clean. They should be prepared by treating in a hot cleaning fluid consisting of sulfuric acid and sodium or potassium bichromate made up according to the formula as given in standard texts. (N.B. After this fluid becomes green or brown it should be discarded.) For best results the covers or slides should subsequently be cleaned in strong caustic soda or potash, rinsed in weak hydrochloric acid, and then in distilled water. They should finally be placed in alcohol and kept there until ready for use. With cover glasses the alcohol can be burned off just before use, while holding each cover with a forceps. In the case of slides, Plimmer and Paine<sup>28</sup> recommend baking on a wire gauze over a Bunsen burner just before using.

The growth should be prepared by diluting considerably in sterile distilled water, and then carefully placing tiny drops or streaks on the coverslip, discarding those coverslips which show signs of grease. The drops or streaks should be so thin as to dry almost instantly; and no appreciable heat should be used in drying them. Some writers recommend drying at body heat, others at room temperature. Fixation of the films by flaming is sometimes specified, but is not to be recommended as it destroys the flagella if they are allowed to become too hot.

Plimmer and Paine recommend the use of slides (scrupulously cleaned), instead of covers. The slide is heated before use and cooled to about body temperature. Then a drop of the culture fluid from a 3 mm. loop is placed at one end of the slide, the slide tilted to allow the suspension to run down the slide. The slide should be warm enough to allow rapid drying. Gray also uses slides, placing a large loopful of the

<sup>27</sup> Johnston, O. P., and Mack, W. B. *American Medicine*, 7: 754, 1904. (Original not seen.) Reference obtained from Moore and Hagan's *Laboratory Manual in General and Pathogenic Bacteriology and Immunity* (1925), p. 99.

<sup>28</sup> Plimmer, H. C., and Paine, S. G. *J. Path. Bact.*, 24: 286, 1921.

culture suspension near one end and making the film by spreading the fluid gently over the slides with a strip of unsized paper (e.g., typing paper).

After the dried films on the cover glasses or slides are ready, the mordant is applied (filtered before use if this is called for by the technique adopted). The mordant is allowed to act between half a minute and three minutes, the time differing with different organisms. In the case of Loeffler's mordant, gentle heat is called for, sufficient to allow visible steam to rise from the solution. The mordants of Casares-Gil, Shunk and Gray are applied cold; Gray allows the mordant to act for ten minutes, although the committee has obtained very satisfactory results with half that period. After rinsing off the mordant the stain is applied in the same way, using gentle heat (enough to cause steaming) in the case of Loeffler's method, but no heat in the case of the other methods given above. The stain is applied from one to three minutes or more. In general, the procedures calling for heat specify about one minute, the others three minutes. Plimmer and Paine recommend five minutes, Gray five to ten minutes.

Other methods of manipulation are sometimes called for, such as dropping the coverslips into test-tubes containing the steaming solutions or by using slides and allowing them to rest on a water bath in which the water is a little below boiling temperature. In this matter, also, the best procedure is undoubtedly the one with which the user is more familiar.

Poor results may be caused by greasy cover glasses or slides, cultures too old or growing on an unsatisfactory medium, allowing the bacteria to stand too long in the dilution water before drying, too thick suspensions of the bacteria, insufficient or excessive mordanting, under-staining, or over-staining. It is very difficult for the beginner to tell which of these points is at fault in a given case, and unless he has expert assistance he can do nothing but proceed by the method of trial and error until chance brings him the right combination. It can be said in general, however, that poor results are more likely to be caused by improper handling of the culture, or by greasy coverslips or slides than by the use of an unsatisfactory staining procedure.

*F. Capsule Stains.* Various methods of staining capsules are given in the literature. None of them, apparently, can be regarded as absolutely reliable. The two following seem to be most used at present in this country.

(1) *Hiss' Method*<sup>20</sup>

Preparations from ascitic fluid or serum media, or other preparations mixed with serum before use, are dried on the slide or cover. The stain

<sup>20</sup> Hiss, P. H., Jr. *J. Exper. Med.*, 6: 317, 1905.

used consists of 5 or 10 c.c. sat. alc. gentian violet or basic fuchsin made up to 100 c.c. with water. This is placed on the dried and fixed preparation and gently heated for a few seconds, until steam arises. The dye is washed off with a 20 per cent aqueous solution of copper sulphate (crystals). The preparation is then dried by blotting.

(2) *Huntoon's Method*<sup>30</sup>

A 3 per cent solution (100 c.c.) of sodium caseinate ("nutrose") is cooked for one hour in flowing steam; 5 c.c. of 2 per cent phenol is added, and the fluid decanted into test-tubes. The organisms to be stained are mixed with a drop of this solution, spread in a thin film on a glass slide, and dried in the air without fixing.

The stain is:

2 per cent aqu. solution of phenol.....	100 c.c.
Concentrated lactic acid.....	0.25-0.5 c.c.
1 per cent acetic acid.....	1 c.c.
Sat. alc. solution of basic fuchsin.....	1 c.c.
Carbol fuchsin (old solution).....	1 c.c.

The stain is kept on the film for thirty seconds, and the latter washed in water and dried.

*G. Conn's Stain for Slime-forming Bacteria*.<sup>31</sup> As explained before, the bacteria which form slime can be handled as ordinary dried smear preparations if the proper stain be used. For this purpose, one of the high fluorescein derivatives such as erythrosin, phloxine or rose bengal proves most satisfactory. The following formula is recommended:

Rose bengal (85 per cent dye content).....	1 gm.
Phenol (5 per cent aqueous solution, by volume).....	100 c.c.
CaCl <sub>2</sub> (1 per cent aqueous solution).....	1 c.c.

This solution is applied cold or preferably with a little heat for about one minute. The slide is then washed very briefly and examined as usual.

### C. STUDY OF BACTERIA IN THEIR NATURAL HABITAT

#### I. In Soil

Bacteria in soil may be stained by the following technique: One gram of soil is mixed with a sufficient quantity of a gelatin fixative to bring the total bulk up to 10 c.c. This fixative is prepared by dissolving 0.015 per

<sup>30</sup> Huntoon, F. M. *J. Bacteriol.* 2: 241, 1917.

<sup>31</sup> Conn, H. J. *J. Bacteriol.*, 6: 253, 1921. Not included in the Manual of Methods for Pure Culture Study of Bacteria.

cent of gelatin in boiling water and using after it is cooled. It is conveniently kept in plugged test-tubes containing 10 c.c. each, which can be sterilized and then used one by one so that the stock of the fixative solution does not become contaminated. The exact proportion of soil and fixative used is not important unless the technique is to be used in estimating the numbers of bacteria present.

About 0.01 c.c. of this infusion is placed on a slide. This quantity should be measured exactly by means of a capillary pipette if an estimate of numbers is to be made; otherwise it may be withdrawn without measuring from the tube, by means of a wire loop about 2 mm. in diameter. The slip used should have been washed in alcohol so as to be moderately free from grease. By means of a stiff wire or needle, a drop of the infusion is smeared over the surface to cover an area of about a square centimeter. If a count of the bacteria is to be made this area should be accurately measured, by means of a square of the specified size placed underneath the slip. If no count is to be made the exact size is immaterial.

The preparation is dried on a boiling water bath. While still thus heated, it is stained for about one minute with rose bengal. This staining solution may be of the formula given before (p. 146). Either phloxine or erythrosin may be used in place of rose bengal, but the writer obtains better results with the latter.

The preparation should be examined with a 1.9 mm. fluorite or apochromatic objective and a 12.5 $\times$  compensating ocular. If it is desired to count the bacteria, the ocular should contain a micrometer bearing a circle divided into quadrants by means of cross lines, and the apparent area of the circle should have been previously determined by means of a stage micrometer.

## II. In Dairy Products

Methods have been proposed for observing bacteria both in milk and in cheese. The former has become a standard procedure and is therefore described at length below. The microscopical observation of bacteria in cheese, however, has been used only for experimental purposes, and is not detailed here. It is a method calling for imbedding in paraffin, sectioning, and staining by procedures that are essentially histological. It is described by Hucker.<sup>32</sup>

For staining bacteria in milk, 0.01 c.c. is withdrawn with a capillary pipette and smeared over one square centimeter of a glass slip as above described in the case of soil infusion. The size of the sample and the area

<sup>32</sup> Hucker, G. J. *N. Y. Agric. Exp. Sta., Tech. Bull.* 87, 1921.

of the smear need not be measured exactly unless the bacteria are to be counted.

The milk smears are dried either at room temperature or with gentle heat; the temperature of a boiling water bath is too high. The slides are immersed in xylol for one to two minutes to wash out the fat. They are then placed in alcohol for about the same length of time to fix the smear to the slip. They are then stained for about a minute in Loeffler's methylene blue solution (which may be prepared if desired with water instead of dilute alkali as a diluent). After staining they are decolorized in alcohol until the smear appears faint blue. After this it is ordinarily found that best results can be obtained by dipping momentarily into the methylene blue again and then washing with water. They are subsequently dried and examined under immersion oil. A lower-powered ocular can be used than in the examination of soil.<sup>33</sup> The ocular should contain a circle of known apparent area as described under soil examination. A fairly accurate estimate of the number of bacteria present in the milk can be made by counting from 10 to 100 fields (according to the abundance of the organisms) averaging the results and multiplying by

$$100 \times \frac{1}{\text{size of field, in sq. cm.}}$$

#### D. SECTIONED MATERIAL<sup>34</sup>

The stains most commonly used for demonstrating bacteria in sections are methylene blue, crystal, methyl or gentian violet and basic fuchsin. Various solutions of these dyes have been devised and are usually known by the names of the men who originated them. The fixatives recommended are alcohol, formaldehyde, corrosive sublimate and Zenker's fluid or combinations of certain ones of them. Zenker's fluid can be highly recommended partly on account of its excellent preservation of the organisms, but also because it furnishes the best fixation of the tissues and of the inflammatory exudation caused by the infectious agents.

From the point of view of staining, the gram-positive and acid-fast bacteria are the easiest to demonstrate because they can be colored differently from the surrounding tissue elements; in other words they can be stained differentially and hence stand out with great sharpness. The gram-negative organisms on the other hand are often difficult to demonstrate satisfactorily. As a result various staining methods have been devised both for this group of organisms and for different members of it.

The following methods can be recommended as the most generally useful and as probably the best which have yet been devised.

<sup>33</sup> Breed, R. S., and Brew, J. D. *N. Y. Agr. Exp. Sta., Tech. Bull.* 49, 1916.

<sup>34</sup> By Mallory, F. B., and Parker, Frederic, Jr.

## Gram-Negative Bacteria

## 1. Mallory's Eosin, or Phloxine, and Methylene Blue Stain.

Zenker fixation, paraffin sections.

(1) Stain sections in a 5 per cent aqueous solution of eosin or in a 2.5 per cent aqueous solution of phloxine for one hour in the paraffin oven at 55 c. or overnight in the cold.

(2) Allow sections to cool for a few minutes and then wash off in water.

(3) Stain in the following solution, using 5 c.c. each of A and B and 90 c.c. of water, for ten to twenty minutes.

*Solution A*

Methylene blue .....	1 gm.
Borax .....	1 gm.
Water .....	100 c.c.

*Solution B*

Azur II .....	1 gm.
Water .....	100 c.c.

Pour the solution on and off the sections several times.

(4) Wash in water.

(5) Differentiate and dehydrate in a dish of 95 per cent alcohol, keeping the section in constant motion, so that the decolorization shall be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still a deep blue, finish the dehydration quickly with absolute alcohol.

(6) Clear in xylol.

(7) Mount in xylol balsam.

For celloidin sections use 95 per cent alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene blue washes it out to a considerable extent. The eosin must be used first, because methylene blue is readily soluble in an aqueous solution of eosin, and therefore is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene blue which is precipitated by any excess of eosin.

The success of this staining method has been found by Wolbach to depend on the presence of colophonium in the alcohol used for differentiation. This is present in alcohol obtained from the barrel, but not in alcohol preserved in glass. It must, therefore, be added. This is most easily done by keeping on hand a 10 per cent solution of colophonium in absolute alcohol, and adding a few drops of it to the alcohol in which the sections are differentiated. Wolbach has also shown that sections fixed in formaldehyde may be stained by this method, provided the

amount of colophonium in the alcohol be increased to from 3 to 10 per cent.

It is sometimes advisable to stain the sections more deeply than usual with the methylene blue solution and then to decolorize for several minutes in a 1 to 1000 aqueous solution of acetic acid before dehydrating in alcohol.

## 2. Giemsa's Method for Staining Bacteria in Sections.

(1) Fix pieces of tissue not more than 2 mm. thick in sublimate alcohol, consisting of two parts of a concentrated aqueous solution of corrosive sublimate and one part of absolute alcohol. The fixation requires at least forty-eight hours. The fixing fluid is to be renewed after twenty-four hours.

The tissue may remain for as long as three months in the fixing fluid without disadvantage if evaporation is prevented.

(2) Dehydrate in graded alcohols and clear in xylol. Imbed in paraffin. The sections should not be over 4  $\mu$  thick; 2  $\mu$  are better. The tissues must not be handled with metal instruments until after they have been cleared in oil of cedarwood.

(3) Treat sections with xylol, followed by graded alcohols and water.

(4) Ten minutes in a solution consisting of KI, 2 gms.; distilled water, 100 c.c.; Lugol's solution, 3 c.c.

Instead of this mixture, it is possible to use Lugol's solution only (1 to 3 c.c. of it mixed with 100 c.c. of water or 70 per cent alcohol), or tincture of iodine diluted with alcohol. The use of the weak alcoholic iodine solution is indicated when a more intense blue staining of the cytoplasm is desired. Treatment with the weaker iodine solutions demands naturally a longer time, twenty to thirty minutes.

(5) Treat with 95 per cent alcohol until the yellow color is removed. After a quick wash with distilled water place sections for ten minutes in a 0.5 per cent aqueous solution of sodium hyposulphite, then five minutes in tap-water, and for a short time in distilled water.

(6) Stain with freshly diluted Giemsa solution two to twelve hours or longer. The solution recommended for this purpose should be made up according to the following modified formula:

Azur II-eosin .....	3.0 gm.
Azur II .....	0.8 gm.
Glycerin .....	125.00 c.c.
Methyl-alcohol .....	375.00 c.c.

The dilution should be 1 drop to 1 c.c. of water; or for a longer period of staining, 1 drop to 2 c.c. of water. After the first half hour the staining mixture is to be poured off and replaced by fresh.

(7) Wash in distilled water and dehydrate as follows

- (a) Acetone 95 c.c. plus xylol 5 c.c.
- (b) Acetone 70 c.c. plus xylol 30 c.c.

(c) Acetone 30 c.c. plus xylol 70 c.c.

(d) Xylol pure.

(e) Cedarwood oil.

(8) Mount in cedarwood oil.

The duration of the treatment with *a*, *b*, and *c* depends upon the degree of differentiation required.

The distilled water used for diluting the staining fluid must be absolutely free from acid. The slightest trace of organic or mineral acids, or even the presence of a considerable amount of carbonic acid, spoils the staining. The distilled water may be tested and corrected for use as follows:

Place 30 c.c. of it in each of four flasks. Add 1 per cent solution of carbonate of sodium ( $\text{Na}_2\text{CO}_3$ ), 1 drop to the first flask, 2 drops to the second flask, and so on. Then take 10 c.c. from each flask in a clean test-tube and add 2 or 3 drops of a fresh solution of hematoxylin in absolute alcohol, which should be pale yellow to nearly colorless. Stand against a white background, and the flask with the right reaction should take on a violet tinge after one to five minutes.

For bringing out certain granules, etc., in special objects a larger amount of alkali in the water is necessary. In this case add to 20 c.c. of the water, shortly before mixing with the staining fluid, an additional drop of alkali solution.

### 3. Wolbach's Modification of Giemsa's Stain.

Zenker fixation; paraffin sections, not over  $5\mu$  thick.

(1) Xylol, alcohol, Lugol's solution; alcohol as usual.

(2) Five-tenths per cent sodium hyposulphite to remove the last traces of iodine, ten to fifteen minutes.

(3) Wash in running water ten minutes, followed by distilled water.

(4) Stain in the following solution for twelve to eighteen hours:

Distilled water.....	100 c.c.
0.5 per cent sodium bicarbonate.....	2 to 4 drops
Reagent methyl alcohol.....	3 c.c.
Giemsa's solution .....	2.5 c.c.

The stain should be poured over the slides immediately after mixing, and should be changed twice during the first hour. The slides are then allowed to remain in the third solution for twelve to eighteen hours.

(5) Differentiate in 95 per cent alcohol.

The procedure of differentiation really consists in removing the excess of stain to a point where good histological detail is secured. If the sections are too blue, a better balance may be secured by adding very small quantities of colophonium to the alcohol.

(6) Dehydrate rapidly in absolute alcohol.

(7) Clear in xylol and mount in oil of cedarwood.



#### 4. MacCallum's Modification of Goodpasture's Method.

Fix in Zenker or Zenker-formaldehyde solution. Use very thin paraffin sections.

- (1) Stain for ten to thirty minutes or more in Goodpasture's fuchsin solution.
 

30 per cent alcohol .....	100 c.c.
Basic fuchsin .....	0.59 gm.
Aniline .....	1 gm.
Phenol crystals .....	1 gm.
  - (2) Wash in water.
  - (3) Differentiate in formalin (40 per cent solution of formaldehyde). Only a few seconds are required. The bright red color changes to rose.
  - (4) Wash in water.
  - (5) Counterstain in a saturated aqueous solution of picric acid for three to five minutes until the section becomes purplish yellow.
  - (6) Wash in water.
  - (7) Differentiate in 95 per cent alcohol. The red reappears and some of it is washed out, as is also some of the yellow of the picric acid.
  - (8) Wash in water.
  - (9) Stain in Sterling's gentian violet for five minutes or more.
  - (10) Wash in water.
  - (11) Gram's iodine solution for one minute.
  - (12) Blot dry without washing.
  - (13) Clear in a mixture of equal parts of aniline and xylol until no more color comes away.
  - (14) Clear in two changes of xylol.
  - (15) Mount in xylol balsam.
- Gram-negative bacteria red, gram-positive bacteria blue; tissue red and blue; fibrin deep blue.

#### 5. The Gram-Weigert Staining Method.

Preferably Zenker's fixation, paraffin sections.

- (1) Stain sections lightly in alum-hematoxylin.
- (2) Wash in running water.
- (3) 1 per cent aqueous solution of eosin, one to five minutes.
- (4) Wash in water.
- (5) Aniline methyl-violet, one-half to one hour.
- (6) Wash off with water.
- (7) Lugol's solution, one to two minutes.
- (8) Wash off with water.
- (9) Blot with filter-paper and dehydrate and clear in several changes of aniline and xylol, equal parts.
- (10) Wash off with xylol.
- (11) Mount in xylol balsam.

When this method is used for staining actinomycetes a saturated solution of eosin should be taken in order to stain the clubs of the organisms sufficiently intensely.

If the tissues have been fixed in alcohol or formaldehyde a carmine stain for the nuclei can be used in place of the alum-hematoxylin and eosin.

The MacCallum-Goodpasture method can also be used for this group of organisms.

### Acid-Fast Bacteria

The tubercle and leprosy bacilli and a few other gram-positive organisms have the property, when once deeply stained by certain aniline dyes, of resisting decolorization by a mineral acid followed by alcohol. A variety of methods have been devised for staining this group of organisms but only the simplest and most generally reliable is given here.

1. **Ziehl-Neelsen Carbol-fuchsin Method.** This is the staining solution most commonly used. It keeps exceedingly well.

Saturated alcoholic solution of basic fuchsin..... 10 c.c.  
5 per cent solution of carbolic acid in distilled water..... 90 c.c.

2. **Ehrlich's Aniline Fuchsin Method.** It is claimed that this solution stains the bacilli more intensely than carbol-fuchsin, but it has to be used fresh because it deteriorates rapidly.

Saturated alcoholic solution of fuchsin..... 16 c.c.  
Aniline water (made by shaking together 5 parts of aniline  
with 95 parts of water and filtering the resulting milky  
fluid) ..... 84 c.c.

The method of using these two staining solutions is the same.  
Zenker fixation preferable; paraffin sections.

- (1) Stain sections deeply in alum-hematoxylin.
- (2) Wash in water.
- (3) Stain in carbol-fuchsin cold over night or steaming for three to five minutes.
- (4) Wash off in water.
- (5) Dip in acid alcohol (hydrochloric acid 1 c.c. to 99 c.c. of 70 per cent alcohol) for thirty to sixty seconds.
- (6) Wash thoroughly in several changes of water.
- (7) Decolorize and dehydrate in 95 per cent alcohol followed by absolute.
- (8) Clear in xylol.
- (9) Mount in xylol balsam.

If celloidin sections are used, they should be attached to the slip by the ether vapor method after the staining with alum-hematoxylin. They can be cleared from 95 per cent alcohol by the blotting paper xylol method.

The leprosy bacillus is more difficult to stain than the tubercle bacillus because it does not retain the dye so well. If good results are not obtained by the preceding method the following procedure can be recommended:

### 3. Flexner's Method for Staining Leprosy Bacilli.

- (1) Stain in alum-hematoxylin so as to get a sharp nuclear stain.
- (2) Wash in water.
- (3) Carbol-fuchsin two to five minutes steaming, or thirty to sixty minutes cold.
- (4) Water.
- (5) Treat on the slip with iodine solution one-half to one minute.
- (6) Water.
- (7) Blot, clear and differentiate in aniline oil.
- (8) Xylol, xylol balsam.

The Gram-Weigert stain gives brilliant results provided the organisms are deeply colored to begin with but of course the method has no diagnostic value by itself.

## CHAPTER IV

### GENERAL BOTANICAL MICROTECHNIQUE

WILLIAM RANDOLPH TAYLOR

(SECTION ON FATS BY SOPHIA H. ECKERSON)

HISTOLOGICAL METHODS 156. Collection, preservation and preparation 156. Reduction of material to physical proportions within range of microscopical observation 165. Preparation and staining of sections 184. Microchemical reactions in plant membranes 195. Mounting methods 197. CYTOLOGICAL METHODS 204. Choice of methods and standards 204. Examination of living cells 211. Preparation of cytological material for fixation 213. Choice of the fixing fluid 215. Imbedding and staining 220. Cytoplasmic inclusions 220. Fats 222. SPECIAL METHODS FOR PARTICULAR PLANT GROUPS 226. Algae 226. Fungi 236. Bryophytes 240. Pteridophytes 240. Gymnosperms 242. Angiosperms 243.

This chapter is intended to be strictly supplementary to the first part of the whole book, where McClung follows specimens of animal tissue through the fundamental preparatory methods.

Both plant and animal materials, however, may be used for introductory training with equal facility. The beginning student should study the first part very thoroughly before consulting this botanical chapter, and all students should beware of assuming that stages in treatment are unessential if omitted or only casually treated here, because processes are not always given in full, but often only their distinctive features emphasized. Cross references to other paragraphs will enable the user to fill important gaps, but a thorough acquaintance with the first part is essential. Stains, for instance, are largely treated separately, and mitochondria and related structures in plants are found in a general chapter including both animal and plant material.

The present chapter treats, first, methods applicable to structures both histological and cytological, found generally in most plants, and then gives special directions which are of particular importance mainly in relation to special plant groups. It does not consider field collecting or the laboratory culture of organisms, and therefore cannot take the place of a Collector's Handbook, or an Encyclopedia of Microscopy. A casual worker will not always be able to refer from the index to a full discussion of the methods of preparing any given sample, for the intention is to offer information and explanations that will facilitate a training in technique rather than a compendium of procedures by which an

untrained operator might be led (fallaciously) to expect that he could, by imitation, duplicate the results of a skilled research student. It is obvious that a broad treatment of this kind can be only a compilation colored by the author's opinions of relative values and needs, however extended his training. Originality of method is not to be sought here, but in research papers. The methods given are comparatively few, and a selection has been made of those that offer advantages in simplicity or precision, discarding those of primarily historical interest or of marked undependability. Limitations of space would have forced this even if it had not been a virtue. The text is not designed for the reference of a skilled investigator, but to supply him his fundamental training, and, should he pass from a familiar to a new field of work, to give him methods by which he might be able to start his investigation and test his material. The most important collateral texts that a student should make available to himself are: Schneider-Zimmerman, *Die botanische Mikrotechnik* 2<sup>te</sup> Aufl., 1922, R. Krause, *Enzyklopädie der mikroskopischen Technik*, 3<sup>te</sup> Aufl., 1926, continued, and C. J. Chamberlain, *Methods in Plant Histology*, Edition 5, 1932, in addition to works on the optics and operation of the microscope and its equipment.

## A. HISTOLOGICAL METHODS

### I. Collection, Preservation and Preparation

1. The general sequence in plant histology through which specimens pass involves their collection, the selection of the appropriate portions, then their removal, perhaps their preservation, followed by detailed manipulations such as sectioning, maceration, etc., which prepare the material for study, and finally the mounting of the ultimate product on slips for observation. The methods suited for vascular plants will be considered here and other groups discussed in special paragraphs. There is little that calls for comment respecting the collection of the specimens in the field. It is obvious that great caution must be exercised to secure representative material in perfect condition. In all serious researches, herbarium specimens from the sources that gave the histological (or equally the cytological) material should always be preserved and filed in permanent collections to permit verification of the original determinations. In fact, portions of the identical specimens should be kept for these records whenever possible, and their relation to the portions studied should be correctly entered on the labels. All specimens, as collected, should be kept appropriately moist and aerated so that they may remain in good health until they can be studied or killed and pre-

served. Just before killing, the portions to be kept for study should be cut free of waste and should be immediately immersed in the preservative. For rough studies in anatomy most material can be sufficiently killed and preserved in 4 per cent formaldehyde solution. For more critical work the following may be used: 70 per cent alcohol without or with 10 per cent of glycerin, formol-alcohol, acetic-formol-alcohol, the mercury fluids, strong chrom-acetic and strong Flemming (p. 217 for formulas and after-treatment). This list gives the less critical fluids first. As one passes to the more precise in action the size of the pieces fixed should be decreased until in strong Flemming the diameter should not exceed 2 to 4 mm., but such fluids are generally used only in studies of tissue differentiation, cell inclusions, etc. In general, this series passes from mixtures giving but poor preservation of the cell contents to ones which, while maintaining the interrelation of the cells, also show correctly the general internal cell structure. After being killed the material should be brought gradually into 70 per cent alcohol, except when one of the first three fluids, which are preservatives in themselves, are used. If the material is to be imbedded before cutting it should be prepared according to the schedules which are given with the imbedding methods (pp. 178, 181). Special preservation methods in histology are too numerous and for the most part too limited in application to deserve consideration here. A few of wide application may be important.

*Calcium carbonate or sulphate deposits* on or in plant tissues do not usually lend themselves to the preparation of permanent mounts. Material should be fixed in alcohol free from acid. Sections of tissues containing, for instance, cystoliths, as in *Ficus* or *Urtica* can be stained and dehydrated quickly, cleared and mounted in balsam, but it is difficult to avoid some solution of the cystolith, which will often dissolve if the sections are left for a little in tap water. (For cryptogams with considerable  $\text{Ca}_2\text{CO}_3$  deposits see p. 222 and the paragraph on the group concerned pp. 229, 234.)

*Cellulose, as food reserve-cellulose* in seeds, often offers great interference to cutting if it is dry and mature. When the conditions of study permit, the material should be gathered and preserved before maturity has completely hardened the walls, and the sections should be cut under water rather than alcohol. Seeds of *Iris* or *Phoenix dactylifera* can usually be managed in this way, the walls appearing practically as thick as if dry seeds had been used. Otherwise, methods applicable to hard woods or coals must be adopted (pp. 174, 182).

*Collenchymatous wall thickening* and pectic jellies interfere with satisfactory cutting if they are too soft. Such material should be preserved and cut under 70 per cent alcohol, swelling if necessary with water after-

wards. It would seem advantageous to harden with even stronger alcohol, but the collenchymatous material sometimes does not expand well after such treatment. In general, soft tissue, if it is to be cut unimbedded will behave best in 70 to 85 per cent alcohol, especially if it has stiff bundles scattered through soft areas, as is often the case with monocotyledonous plants and ferns.

*Crystals as cell inclusions* (usually calcium oxalate, occasionally the sulphate) show by far the best in fresh-cut sections from living material. However, they may be mounted in 2 per cent acetic acid and will keep for a time very satisfactorily (*Dieffenbachia* stem, *Allium* bulb coats, etc.). When carried through into balsam they generally lose in visibility from too close approximation to the refractive index of the medium.

*Herbarium specimens* or stored dried crude vegetable drugs are often called upon for histological material. The lower groups of plants are considered separately, but certain general rules apply to them and to the vascular plants in common and may be discussed here. In general the parts desired must be cut away with great caution. This is particularly the case in studying plants in public or borrowed herbaria, for from such no portion should ever be removed except for grave causes and after specific permission. The fragments, if desired for microchemical studies, must receive special treatment depending on the character of the substances to be retained. For ordinary purposes they can best be moistened with alcohol to reduce the air film, then soaked up with water, or boiled if necessary to hasten the process. Lactophenol is excellent to soften specimens, as is lactoglycerin with a little water, and dilute caustic potash may sometimes be used (p. 198). Even if it is desired to macerate (p. 174) the tissues for epidermis, bast or wood fibers, idioblasts, etc., it is best first to soak it up thoroughly to assure even action of the macerating fluid. The same methods are used, after the material is soft, as are applied to material preserved in liquid, and in many cases the results are equally satisfactory. Herbarium material may also be imbedded for histological studies. Very thorough soaking should precede the dehydration, and the use of a vacuum pump to remove air is especially advantageous. The usual methods are applied (p. 179).

*Inulin* can readily be retained in the tissues if the material when freshly collected is cut into about 1 cm. blocks and treated with several changes of absolute alcohol, to remove the water rapidly and completely. Sections must be cut with a knife flooded with absolute alcohol. The precipitated sphaero-crystals of inulin may be observed in absolute alcohol, redissolved in water, or the dehydrated sections may be stained with orange G dissolved to saturation in clove oil, washed in xylol and

mounted in balsam, the inulin being darker in color than the general tissues.

*Latex-containing plants* should be collected in such fashion as to prevent the loss of too much fluid before the fixation is effected. Where there are long latex vessels "bleeding" may be prevented by ligating the stem near the end of the piece to be cut away. It is preserved in 50 per cent alcohol without the removal of the ligature or clamp until hardening is complete. For further treatment see p. 194.

*Resin-containing material* (e.g. gymnosperms) should be immersed in a saturated aqueous copper acetate solution for a considerable period (one to several weeks) after which the excess copper may be washed away and the material preserved in 50 per cent alcohol. Sections are reported as retaining the bright green coloration of the resin when mounted in glycerin.

*Sieve tubes* (p. 194) containing a considerable quantity of coagulable protein substance are usually satisfactorily shown when put up in alcohol, but the distribution of coagulum within the mature tubes is not always correctly maintained. It is best to immerse the plant to be studied, or a long unsevered branch of it, for a few moments in boiling water to coagulate the contents before cutting it up into lengths suitable for preservation.

*Starch-containing material* should be preserved in alcohol rather than formalin, or with acids which hydrolyze it, if the retention of the starch in good condition is desired. In most histological work it can well be dispensed with.

2. **Preserving media for plant tissues**, while offered in a wide variety of forms, are founded on comparatively few basic formulas. The safest general purpose preserving fluid is 70 per cent ethyl alcohol. To minimize loss by evaporation 5 to 20 per cent of glycerin may be added. While the strength of the solution may be reduced to 50 per cent alcohol for tough specimens destined for superficial morphological study it should be increased to 85 per cent for exceedingly delicate material or material for cytological study. Glycerin is generally to be avoided if the material is later to be imbedded in celloidin or paraffin, and if present should be removed by very careful washing before the final stages in dehydration. Woody material may be preserved in n-butyl alcohol with a minimal increase in brittleness. Filamentous algae and fungi are advantageously transferred from the wash water, after fixing or staining, to 5 or 10 per cent glycerin which is concentrated by evaporation, the concentrated glycerin serving alone as a very good preserving fluid. A general fixing and preserving fluid consists of 70 per cent alcohol 96 parts, formalin 4 parts. A recently suggested fluid contains 50 per cent



ethyl alcohol 100 c.c., formalin 6.5 c.c., glacial acetic acid 2.5 c.c. It is probably better than the preceding, and is very good as a histological fixing agent, preserving the cell contents tolerably well. Formalin alone in 4 per cent aqueous solution has a general application for all tougher material intended for morphological studies and keeps it in a less brittle state than strong alcohol. It should be used with great caution on segmented Rhodophyceae, since these often disarticulate after a few months. This can be avoided in part by storing in the dark and using formalin neutralized by the addition of borax until the solution gives a red color with phenolphthalein, which also will serve for the preservation of calcified organisms, although if possible these should be kept in alcohol. The addition of cupric acetate to saturation in the stock formaldehyde solution will yield on dilution to 4 per cent a mixture which will preserve the green color of plant parts very well. Coarse specimens such as equisetums, mosses and ferns for the best results should be placed first in a hot solution for a few minutes and then removed to a cold solution. Preservation in Stromsten's fluid (p. 219) is also effective. Methyl alcohol may frequently be substituted for ethyl when the latter is unavailable, but denatured alcohol should be avoided if possible because of the cloudiness of the aqueous mixture.

A formula offered by Keefe is notably free from any tendency to shrink and maintains a natural green color: 50 per cent alcohol 90 c.c.; commercial formalin 5 c.c.; glycerin 2.5 c.c.; glacial acetic acid 2.5 c.c.; copper chloride 10 gm.; uranium nitrate 1.5 gm. This serves as a fixing and a preserving fluid combined. For Myxophyceae 10 gm. of copper acetate may be substituted for the copper chloride plus uranium nitrate. The presence of alcohol will prevent the use of this fluid in microscopic mounts but the material may be transferred to a mixture from which it has been omitted. Material stored in it should not be exposed to sunlight.

3. **Decalcification of plant tissues** must be conducted in a gentle fashion as it is of first importance to avoid distortion due to the liberation of gas or to the direct action of acid on the tissues. For morphological studies of most plants a dilute solution of hydrochloric acid (2 per cent) is suitable, but for more rapid results a 10 per cent solution may be used. The solution should be changed freely until all lime has been dissolved. For more gentle action acetic acid may be substituted; nitric acid is also used, but is rather violent.

If a more accurate preservation is desired it is well to effect decalcification by the use of one of the common fixing fluids, such as chrom-acetic, Flemming, or sublimate-acetic. The solution must be used liberally, and in the case of Flemming the later stages of decalcification

may be completed with chrom-acetic if the amount of osmic acid that would be consumed is an important item. If gas tends to collect in intercellular spaces it will interfere with the action of the acid, and even distort the tissues, and should be frequently removed by the use of an air pump. If the material is rather soft it may be possible to fix with formol-alcohol and decalcify after hardening in strong alcohol by the gradual addition of acetic acid.

Formaldehyde, gradually decomposing to formic acid, will eventually reduce or remove the calcification from stored specimens, so that if the deposit is not great it may soon be dissolved. This is particularly noticeable in the case of slightly calcified tropical algae, which after two to three years may be completely decalcified. For means of preventing this see p. 160. Some phycologists find Perenyi's solution (p. 559) an acceptable decalcifying agent. (See also Bone and Teeth, pp. 346, 360.)

4. **Desilicification of plant tissues** is generally effected with hydrofluoric acid. The specimens to be treated are brought into a wax vessel or a paraffin coated bottle and flooded with acid, full strength if the specimens are heavily impregnated, or diluted to 50 per cent if they are softer. Dry woods must be boiled thoroughly and exhausted of air under an air pump before introducing the hydrofluoric acid. Capsules of mosses, etc., must be punctured with a needle to let air escape. The action may take several weeks, and the container should be kept out of doors (as on a windowsill) and not in a laboratory, since the fumes are detrimental to apparatus. A new proprietary fluid, Diaphanol, has recently met with some favor as a substitute for hydrofluoric acid. It may be used for indurated tissues in general, both lignification and chitinization being amenable as well as silicification. It is suited for use on partly carbonized peats, producing a bleaching as well as a softening effect.

5. **Dehydration and clearing of plant material** is to be conducted with regard to the condition of the material, bulk or section in form, and to the purpose, histological or cytological, to which it will be put. Most material of plant tissues destined for histological studies will either have been fixed in formol-alcohol or some mixture requiring little if any more attention than washing and preservation in 70 per cent alcohol. Material fixed in strong chrom-acetic may, after washing, deserve dehydration through 15, 25, 50 to 70 per cent alcohols with three to six hours in each change. For work on most root tips and on the development of the sporangial structures in phanerogams and ferns the series should be extended. If it has not been possible to actually lay bare the archesporangium it is not often worth while to extend the series unduly. For such, gradations of 10 per cent intervals serve all practical needs and the time of immersion may vary from fifteen minutes (small, uncuticularized ob-

jects, as delicate root tips) to one to two hours (large ovaries, young cones, etc.). When serious cytological work is contemplated on dermatogen cells of root tips or archesporia which have been laid bare before fixation, or on easily permeable algal or fungal material, the series can advantageously be made more gradual with a 2.5 per cent initial stage and after 5 per cent, then 5 per cent gradations to 30 per cent and 10 per cent gradations to 80 per cent. Marine organisms should sometimes have an alcohol dilution series based on sea water (p. 164). See also the smear methods for plant cytology (p. 165).

Recently there have been suggested several substitutes for ethyl alcohol in this process. Dioxan<sup>1</sup> appears to be the best of these. This fluid is miscible with water, ethyl alcohol and paraffin. Used fluid will give up admixed water and alcohol to anhydrous calcium chloride and when so rejuvenated may be used for a first treatment of the material. The fresh dioxan used before paraffin should be kept dry over calcium chloride, but in this condition readily permits its replacement directly by melted paraffin in the usual way. (See Chapter I, p. 14.)

Dufrenoy reports success both on woody tissues and on delicate protoplasmic structures with the use of methylol,<sup>2</sup> which is used first in equal parts with water, then full strength, then dehydrated with anhydrous sodium carbonate, finally in equal parts with paraffin oil. From the oil transfer to soft paraffin direct offers no difficulty, and the whole process may be completed in three hours.

Zirkle has recommended the use of N-butyl alcohol<sup>3</sup> for hard or brittle subjects. It is necessary to use ethyl alcohol in the lower mixtures, so that the process is somewhat more complicated than the foregoing:

Distilled Water	Ethyl Alcohol	Butyl Alcohol
50 per cent	40 per cent	10 per cent
30	50	20
15	50	35
5	40	55
—	25	75

Two or more changes of absolute butyl alcohol precede transfer to paraffin, which mixes with the butyl alcohol but slowly, and effectively only at the temperatures of melted paraffin. The butyl alcohol with the specimens should be stratified over paraffin, and after the specimens have sunk to the bottom of the wax the supernatant liquid should be poured

<sup>1</sup> Graupner, H., and Weissberger, A. *Zool. Anz.*, 96: 204, 1931.

<sup>2</sup> Dufrenoy, J. *Science*, 82: 335, 1935.

<sup>3</sup> Zirkle, C. *Science*, 11: 103, 1930.

off and replaced with fresh. The dehydration stages will require about an hour each for material of ordinary penetrability, and the stages of infiltration about twelve to eighteen hours for the first stages and six hours for the last stage.

*For filamentous organisms* it is often advantageous after washing, simply to place them in 5 per cent glycerin, which is then concentrated by evaporation in a warm place (as above a radiator) protected from dust. When it is thick and syrupy, material can be stored in it, or for imbedding the glycerin can be washed out with 95 per cent alcohol. This method can be extended to bulkier tissues (root tips etc.), but with questionable advantage.

*The dehydration of sections* is almost invariably effected by passing them through a graded series of alcohols. The length of the series is determined by the critical nature of the material. For ordinary histological studies a sequence of 30, 50, 70 and 95 per cent alcohol should be quite enough, and often 50 to 95 per cent will serve. For cytological studies the sequence must be more detailed, and care must be taken that the surface fluid remaining on the slide from one change is really replaced by the higher grade, by dipping the slide several times, or long immersion. Dehydration of sections (not in celloidin) is always completed with absolute alcohol unless a clearing fluid of special character is used. In extreme cases the slides may be laid in flat dishes and dehydrated by the concentration of weak glycerin. Generally a sequence of 5, 10, 20, 30, 40, 50, 60, 70, 83 and 95 per cent ending with two or more treatments with absolute alcohol is quite correct. More wide-spaced changes are justified only when staining with aniline dyes that come away readily, and are to be looked upon with suspicion when dealing with work of anything like critical character. Carbolic acid and glycerin can also be used for dehydration purposes if occasion requires. Dehydration of intact specimens, as also of sections, can usually be accomplished by the use of fewer changes than when removing the water, as the tendency is to swell rather than to shrink the cells.

Dehydration can be effected from the water-saturated stage by immersion of the material in liquefied (aqueous 90 per cent) carbolic acid. This is a rough treatment, but suited to the clearing of leaves, etc., destined for whole, unstained mounts. Thin sections containing alcohol- or water-soluble crystals, etc., can frequently best be dehydrated simply by drying on the slide.

*Clearing of sections of plant material* is necessary between the alcohol and the mounting medium stages. A graded series of absolute alcohol-xylol mixtures is the best method, though hardly the least troublesome. For plant material the series should be almost as long as for the dehydra-

tion of the sections. After 2 to 3 changes of pure xylol the material may be placed in the resinous mounting medium. Cedar oil may be used to clear sections from absolute alcohol, but is rather viscous and easily clouds with moisture from the air. Clove oil may be used readily after absolute alcohol and will clear from 95 per cent alcohol or even lower; it is of great service in cytological and histological work, but for the former cannot compete in delicacy with an extended alcohol-xylol series. A mixture of 2 parts of red oil of thyme with one part of oil of cloves is better and cheaper than pure oil of cloves; it does not destain quite as rapidly. Aniline oil may be used to clear from alcohols of as low as 90 to 95 per cent, but is hard on stains and of no cytological value. For use with celloidin a mixture of carbolic acid (crystalline state) and xylol (about 25 per cent of carbolic, or less) is best, as it clears easily and safely from 95 per cent alcohol, and does not seem to hurt stains. Eycleshymer's fluid is sometimes ruinous to stains. After any of these clearing fluids it is necessary to wash out thoroughly with xylol, as most of them have some effect on the dyes or the mounting medium. Benzol and toluol may be substituted, if desired, for xylol in most procedures.

*Clearing of plant material in bulk* for paraffin imbedding is effected with the same substances that are used for sections. The best, by far, are cedar oil, and xylol in an absolute alcohol-xylol series in which the strength intervals must be closer than for sectioned material, and the periods of immersion longer; perhaps half an hour in each grade for root tips. With cedar oil it is customary to place some oil in a vial and an equal volume of absolute alcohol containing the material above it. As the objects sink through the surface of the oil they become gradually impregnated. When on the bottom the specimens should be washed for some time with fresh oil, then washed clean of it in xylol, and infiltrated. The alcohol-xylol series is much the most precise.

*Dehydration of marine plant material* may be somewhat complicated by the presence of dissolved salts. While it is generally easiest gradually to replace a fixing fluid with fresh water after killing marine plant tis-

Water, Fresh	Water, Salt	Alcohol
5 parts	90 parts	5 parts
10	80	10
20	65	15
30	50	20
35	35	30
40	20	40
50	.....	50

sues, it is sometimes desired to dehydrate immediately after washing with salt water, or without previous washing. In such cases the preceding table of fresh-salt water mixtures will generally enable the material to be brought into 50 per cent alcohol without shrinkage or cloudiness, but intermediate stages may be interpolated for most critical studies.

## II. Reduction of Material to Physical Proportions within Range of Microscopical Observation

### 1. Non-Section Methods.

*a. Smearing. The acetocarmine method* as adapted to plants by Belling<sup>4</sup> is unsurpassed for immediate results in making chromosome counts and other observations of an urgent nature. The preparations secured are essentially temporary, although they may be converted to semipermanency. More than one schedule is available.

The acetocarmine stock is prepared by boiling an excess of powdered carmine in 45 per cent glacial acetic acid. In a drop of this the anthers are teased out with steel needles and mounted. The chromosomes will be reddish, darkening or keeping to purple. A second schedule calls for the addition of a trace of ferric hydrate solution to the stock acetocarmine, stopping short of the production of a precipitate. Then dilute with an equal quantity of unmodified acetocarmine. Tease out the anthers with nickel instruments. The third schedule involves fixing the anthers in a mixture of 1 part of glacial acetic acid to 9 parts of absolute alcohol which has been colored brown by ferric hydrate (the amount varied to produce the best results). After days, or even weeks, the anthers are teased out in the original acetocarmine with nickel instruments.

This method has been enthusiastically adopted and with it many valuable results have been obtained. As the cytoplasm swells after a time the chromosomes can be squeezed apart from each other by pressure on the cover glass and this often facilitates counting. Belling recommends<sup>5</sup> the use of water-immersion objectives and of green light screens (Wratten filter 57a or 58) in the observation of acetocarmine preparations. How much more than the count can be effectively studied with this method is uncertain to the present writer. A method designed to give permanent preparations has been elaborated by McClintock<sup>6</sup> according to which anthers are collected and placed in a solution of one part glacial acetic acid to 3 parts of absolute alcohol. They are best used after twelve to twenty-four hours, but may be left in this solution for several

<sup>4</sup> Belling, J. *Amer. Nat.*, 1921.

<sup>5</sup> *Ibid.*, 1923.

<sup>6</sup> McClintock, B. *Stain Technology*, 4: 53, 1929.

weeks. To use, the contents of the anthers are squeezed into a drop of Belling's iron-acetocarmine mixture. When all fragments of anther wall and other debris and covering are removed, the slide is to be passed over a flame for a second 4 or 5 times. If the slide is then put into a Petri dish of 10 per cent acetic acid the cover glass will soon separate from the slide. Both slide and cover are then to be run through successive solutions consisting of 25 per cent, then 10 per cent glacial acetic acid in absolute alcohol in turn, followed by absolute alcohol and then absolute alcohol and xylol to pure xylol for mounting in balsam. This same investigator<sup>7</sup> has found that if the slide after heating is subjected to careful pressure on the cover glass the chromosomes even in early stages will separate and spread out so as to be much more readily studied, though it may not be possible to make such smears permanent.

A method which is rather more precise has recently been tried extensively by Steere.<sup>8</sup> In this, test anthers are stained in hot acetocarmine in the usual way; the anthers promising satisfactory stages are smeared by Taylor's method (p. 168) but fixed inverted on steaming hot acetocarmine solution. After one to ten minutes they are transferred through 66 per cent glacial acetic acid in absolute alcohol, 33 per cent glacial acetic acid in absolute alcohol, pure alcohol, alcohol-xylol mixture and pure xylol to be mounted in balsam. The process gives dark red or purple chromosomes on a nearly colorless background in five to fifteen minutes.

*The hematoxylin-balsam smear method*, yielding permanent results, was adapted to plant material by the writer some few years ago. Microspore mother-cells were so "smeared" as to cause the cells to adhere to the slip without any artificial cementing agent, but sufficiently strongly that they could be stained and mounted in position. Originally designed simply to facilitate chromosome counting, it became evident that the method was fitted to give results that would establish quite new standards in preparations of nuclear structures. Various types of temporary preparations have been developed (p. 165) which serve tolerably for making chromosome counts, yet none seem worthy of serious consideration in studies on the critical prophase and telophase stages or on chromosome structure. Those based on acetic acid, especially, are objectionable for their distortions of chromosome shape and of the spireme. While the chromosomes retain many of their structural features (constrictions, etc.) they are not usually accurately represented. The method developed by the writer retains all the best features of the invaluable chromo-acetic fixation method. It applies this fluid under the only con-

<sup>7</sup> McCintock, B. *Proc. Nat. Acad. Sci.*, 16: 791, 1930.

<sup>8</sup> Steere, W. C. *Stain Technology*, 6: 107, 1931.

ditions in which it can perform its best service, namely, with the important cells directly exposed to the unimpeded action of the fixing solution, and with almost instant contact of cells and solution. The cells after fixation can be washed, stained, dehydrated and mounted in balsam by Heidenhain's hematoxylin technique, the most accurate devised for nuclear studies. Shrinkage of the protoplast is enormously reduced (often apparently eliminated) and this is not merely due to the unbalanced swelling action of acetic acid. In addition to the orthodox technique this method offers by far the most crucial opportunity for testing other fixing fluids and their variants or adjuvants, because since the cells studied are directly exposed to the fluid there is no question as to the filtering action of superposed cell layers (anther walls, etc.). The precision of response to changes in the fluids is much greater by this method than by any other. By it the writer and his students have been able to secure most valuable data on the structure of the spireme, to demonstrate the chromosome tetrad in the late prophase and the structure of the chromosomes, to determine most accurately the constrictions related to the spindle fiber attachment, and to reduce or even eliminate the contraction feature which was supposed to be an essential element of the synizesis (synapsis) stage, provided the material is in such a state as to permit smearing at this meiotic period. The chromosomes show far less tendency to clump than in imbedded and sectioned material. The cells can be scraped loose from the slide and caused (by local pressure) to rotate in the balsam under observation so that all views may be seen of a single meiotic or mitotic figure. The mounts are, of course permanent records, which is not the case in the glycerin or acetic methods. As was noted when the process was originally described:

"Because the cells are spread out in a single layer and in immediate contact with the fixing fluid, the quality of the fixation may be very high. The karyolymph is precipitated in a uniform and delicate way, giving the much desired 'solid fixation' of the nucleus, as a result of which the chromatin elements retain their normal position within the membrane, resisting the tendency to clump at various stages so familiar in paraffin material. The cells are entire, and the observer may work without the necessity of accounting for parts of one cell displaced in two or three sections. This is not always an advantage, but in making chromosome counts it is much to be desired, and this method gives entire metaphase plates without broken or cut chromosomes. It might be thought that the making of the smear would rupture the pollen mother-cells, or at least displace the contents within them, but it is found that most of the cells escape damage, and in the others it shows so obviously that there is almost no possibility of confusing normal and abnormal conditions."

The method has been used in extended studies on *Gasteria* and



*Veltheimia* by the writer, and by Kaufmann (p. 170) on *Tradescantia* (*Rhoeo*) and *Podophyllum*. It has also been used on several other forms, such as *Haworthia* and *Pitcairnea*.

The use of spring rye has proved very advantageous for instruction in this technique. The chromosomes are few and large. The plants can be matured in pots in the greenhouse in very few weeks and the heads show sufficient range of stage to make them convenient for student use. Test samples should be taken well before the heads leave the sheaths. In the winter they will be ready in about ten weeks and in the summer in about four weeks after planting (Davis). The genus *Gasteria* with its long racemes furnishes an abundance of easily grown material with a wide range of stages, but the plants must be carried over from year to year, and the season is of but a few weeks' duration.

The important limitations of the method are, first, that it can be applied only to cells not joined together by a firm middle lamella. In higher plants this means, essentially, spore or microspore mother-cells after they have begun to round up (the beginning of the breakdown of the archesporium) although occasionally a fairly intact archesporium may smear tolerably well. Secondly, the thick jelly-like wall around the microspore mother-cells and the quartets (tetrads) interferes with the use of aniline dyes as counter-stains, although safranin and some others can be used as satisfactory single stains. It does not succeed well on species which are heavily gorged with stainable food material during the maturation divisions, or on pollen grains after the coagulable material in the anther sacs has disappeared.

The method was originally described in part as follows:

"The fixing fluid used is a chrom-osmic-acetic mixture of the following constitution: 10 per cent acetic acid 2 c.c.; 10 per cent chromic acid 0.2 c.c.; osmic acid dissolved to 2 per cent strength in 2 per cent chromic acid 1.5 c.c.; distilled water 8.3 c.c. About 1 per cent of maltose has usually been added to this fluid, which will probably have to be modified to suit each plant on which it is used. A slender glass rod is laid in the bottom of a Petri dish, and enough of the fixing fluid poured in to cover it. It has been found more convenient to use slides than cover glasses, and these are cleaned by long immersion in battery fluid, rinsed, and dried with an absolutely clean cloth. The anthers are excised, and as soon as collected are crushed and spread over the center of the slide with quick strokes of a clean scalpel, which must be honed flat and smooth on the face or the smear will not be a success. The slide must be immediately inverted on the fixing fluid, bringing it down in a horizontal position, so that the whole smeared face is wet simultaneously. The time from the first crushing of the anther to fixing should not exceed three to five seconds. If the slide is brought down on the fluid in an oblique position much of the material will wash off. The slide may stay in the Petri dish, resting on the glass rod for a few minutes, while a second is prepared and placed beside it; then it may be removed, turned right

side up, flooded with the fluid, and left for the full time of fixing, or about fifteen minutes. The slides are then to be washed in changes of water in staining wells for about an hour, and any large pieces of anther walls, filaments, etc., which have not broken loose, can be picked off with a needle. A preliminary inspection of the material also can be made, and slides showing stages which are not desired may be discarded. The developing pollen mother-cells adhere excellently, and rough rinsing does not dislodge them. Smears of pollen grains are quite easily made, so long as they are still surrounded by viscous fluid in the anther, and a very fair fixation may be secured through the chitinous wall, so that the first mitosis within the pollen grain may be critically studied.

"If the slides are to be stained at once, they are then bleached in diluted aqueous hydrogen peroxide, rinsed again, and placed in 2 per cent iron ammonia alum solution; or they may be partly dehydrated and held in 70 per cent alcohol for later staining. After four to twelve hours in the iron alum they are again washed most carefully for fifteen minutes or more in running water, and stained for four to twelve hours in 0.5 per cent aqueous haematoxylin solution, followed by another rinsing, destaining under observation in the iron alum, and an hour of final washing. Dehydration is to be accomplished gradually through alcohols by 10 per cent stages, but the slides need remain in each stage only two to three minutes. Clearing must be done by mixtures of xylol and absolute alcohol, of which about four intermediate stages seem to be enough. In this connection it must be noted that the somewhat mucilaginous walls of the developing pollen mother-cells are intact, and as a result any great change in the density of the successive solutions into which the slides are introduced is sure to cause collapse of the walls and shrinkage of the protoplast. After the smears have come from pure xylol, very dilute xylol balsam is dropped on them and allowed to concentrate by drying for a few minutes before the cover glasses are put in place.

"The chief difficulty encountered is that of getting a brilliant stain, but the writer has eventually succeeded in doing so in those plants which he has so far tried. The haematoxylin and iron alum must both be of the best grade and in good condition, but in spite of care some batches of slides will show a muddy stain. Passable results can be secured within a single working day by shortening the staining periods, thus enabling chromosome count work to be done very rapidly, with the advantage over the acetocarmine method of permanency of the slide records." McClung<sup>9</sup> has published critical studies of fixative action on smears of animal tissue.

The use of alcohol alone as a fixing fluid for plant material is not generally to be advised except for simple morphological studies. However, it has been used successfully for smears by Yasui<sup>10</sup> who found that a concentration of between 75 per cent and 95 per cent was most effective, and saved much time by avoiding the washing and dehydration stages. It is recommended for use before Fuelgen's stain rather than for critical structural cytology.

<sup>9</sup> McClung, C. E. *Anat. Record*, 14: 265, 1918.

<sup>10</sup> Yasui, K. *Cytologia*, 5: 140, 1933.

Working in the present writer's laboratory, Kaufmann has studied the adaptation of this smear method to a variety of materials, and has recently offered a very valuable modification of it. His results demonstrate a remarkably consistent spiral structure in meta- and anaphase plant chromosomes and enable a fairly continuous interpretation of the mitotic cycle to be presented. He suggests the following plan<sup>11</sup>:

"The present writer [Kaufmann] encountered the same trouble [occasional muddy stain] in early efforts to secure consistently good results, but the following alterations in the staining schedule increased materially the proportion of usable slides. Instead of mordanting in 2 per cent iron alum for a period of four to twelve hours, as suggested by Taylor, the same concentration was used for forty-five minutes to one hour. Washing in running water followed for about ten to fifteen minutes. The staining process was curtailed to twenty or thirty minutes in a 0.25 to 0.50 per cent solution. The necessary time can be estimated after a few trial experiments by the color concentration in the cells. As soon as a deep purplish-black appeared it was found advisable to transfer the slides to water. Differentiation in the 0.50 per cent solution of ammonioferric alum then could more often be completed prior to the appearance of a muddy color than was possible with the prolonged staining.

"The iron alum was diluted as desired from a 20 per cent stock solution. The haematoxylin was of American manufacture and certified by the Commission on the Standardization of Biological Stains. No advantage seemed to accrue by allowing the solution to 'ripen' for any considerable time. Often the crystals were dissolved not more than one hour prior to the time of staining. The solutions were used but once or twice as a further caution against the muddy color.

"... The curtailed periods of staining facilitate the handling of a considerable number of slides in the course of a day. If a suitable plant is available which shows active cell division during the morning hours, it is possible to smear 40 or more slides at that time and to present the permanent mounts by evening. The writer has found a flat honed scalpel with a straight cutting edge the most advantageous for making smears. Small anthers like those of *Tradescantia* and *Rhoeo* can be crushed and the contents spread with one stroke of the scalpel. While spreading the cells an effort is made to increase gradually the pressure on the scalpel. In this way some cells will usually be found deposited in but a single layer, although others may be crushed. Some excellent preparations of chromosome structure have been obtained at that intermediate zone where the pressure on the scalpel was great enough to rupture the cell wall but not sufficient to destroy the protoplast. Success in preservation is due evidently in these cases to the instantaneous penetration of the fixative.

"... Of the various fixatives employed the chrom-osmic-acetic acid and the picric-acetic-formaldehyde combinations proved the best. In most cases penetration was enhanced by the addition of such adjuvants as lactose, maltose and urea

<sup>11</sup> Kaufmann, B. P. *Stain Technology*, 1927.

in concentrations ranging from 1 to 5 per cent. It is impossible, however, to detail a single formula which proved adaptable to all phases of meiosis. For example, Bouin's fluid plus maltose was excellent for preserving the later prophase stages of the first maturation division but was not so adaptable to other phases."

Newton<sup>12</sup> has recommended the use of gentian violet as a stain, followed by dehydration in alcohols containing 1 gm. per cent each of iodine and potassium iodide, whereby it is possible to obtain stains of any intensity required, while the transparency of the cytoplasm renders easy the examination of thick sections or smears. The gentian- or crystal-violet stain as applied to a fixed smear may be superbly differentiated, as shown by an example (*Tulipa*) kindly sent to the present writer, who has in confirmation also secured very fine differentiation on root tip (*Allium*, *Fritillaria*) material. If destaining with iodine solution is too slow it may be alternated with clear alcohol. Observation of such slides is further facilitated if a Wratten B green screen is used below the microscope condenser, whereby the stain appears almost as black, clean-cut and sharp as in a hematoxylin preparation, or they may be used with a Wratten K<sub>3</sub> yellow screen, when the stain appears a deep, sharp and rich red.

A modification of this process has been suggested by F. H. Smith<sup>13</sup> in which the rather extreme transparency which is sometimes unavoidable may be mitigated and fading tendencies reduced. The slides are mordanted after dehydration with a solution composed of 1 per cent each of iodine and potassium iodide in 80 per cent alcohol. They are then removed directly to water and stained in filtered aqueous saturated crystal violet for five to ten minutes, again rinsed, treated directly with the iodine solution, rinsed in 95 per cent alcohol and rinsed in absolute alcohol saturated with picric acid and clear absolute alcohol in turn. Differentiation is effected in clove oil. The extreme changes between strong alcohols and water limit the usefulness of this method.

*Tuan's Safranin and Picric Acid Technique.* There has always been considerable difficulty in differentiating safranin O stain sharply and eliminating all muddiness from the cytoplasm, particularly in smear preparations. Tuan<sup>14</sup> has suggested the use of picric acid as a controlling agent in the destaining process, and this gives very dependable results. Smears fixed by Taylor's method (p. 168) are washed, bleached, washed again and stained in sat. aq. safranin O for five to ten minutes, and again rinsed in water. Dehydration is begun with 10 per cent alcohol saturated with picric acid, and completed with a long series of alcohols each with

<sup>12</sup> Newton, W. C. F. *Linn. Soc. J. Bot.*, 1927.

<sup>13</sup> Smith, F. H. *Stain Technology*, 9: 95, 1934.

<sup>14</sup> Tuan, H.-C. *Stain Technology*, 5: 103, 1930.

1.5 per cent picric acid. The picric acid is removed in the neutral 95 per cent alcohol stage by adding a small amount (less than 0.5 per cent) of ammonia water, and immediately washing with pure alcohol. After this the slides are prepared as described in that smear method for balsam mounting. Sections should have a longer staining period, about thirty minutes to three hours, and should have five to ten seconds in each alcohol instead of about two minutes. If the stain is too dark the time in the alcohols may be increased. The chromosomes in the slides prepared by this process should be brilliant red and the cytoplasm colorless. If the ammonia stage is omitted the cytoplasm may remain yellow.

*Backman's Alizarine Fixing and Staining Method.* There is much demand for a method which will yield stained preparations suitable for making chromosome counts in the shortest possible time which will also be essentially permanent in nature. The solution organized by Backman<sup>15</sup> for this purpose is compounded as follows: Anthraquinone, sat. aq. sol., 75 c.c., formaldehyde, 25 c.c., acetic acid, c.p. 99.5 per cent, 5 c.c., alizarine red S, 0.125 gm., saponin 0.15 gm. Before using this it is necessary to add 8 to 12 drops of a normal solution of a metallic salt. The tone varies with the salt chosen, and aluminum chloride, hydrated copper nitrate and ferric chloride are all suitable. Just as much salt should be added as the mixture will hold without precipitation of the hydroxide while the solution is in use. Smears were prepared according to Taylor's method (p. 168) but fixed three to five minutes in the solution given above, washed in tap water two to five minutes, dehydrated through the long alcohol series for one minute in each stage, and differentiated with 0.5 per cent alcoholic sulphuric acid saturated with picric acid until the chromosomes are defined, this usually being accomplished in a few seconds. The slides are then washed with 95 per cent alcohol. The stain becomes darker with time, but if it is desired to hasten the process the addition of 4 per cent cymene to the last alcohol will accomplish this. The smears are then prepared for balsam through the longer absolute alcohol - xylol series. Sectioned material may also be prepared by this method, but the staining may take six to eight hours. Smears may be made ready for study in a very short time, so that a hundred slides prepared in sequence may be carried through the process in two hours while the Newton method requires about six for the same number. A green screen used before the light when examining slides stained by this method will increase the visual contrast afforded.

Since the procedures outlined here are rather new to botanists they have been given in detail in order that no lack of reasonable suggestions should prevent their wide and careful trial. They do not relieve the

<sup>15</sup> Backman, E. *Stain Technology*, 10: 83, 1935.

technician from the necessity of intensive effort in mastering the method. For its finer results this smear method calls for a building up of personal skill quite equal to that required for mastering the paraffin method, and must be attained in part independently of a knowledge of that method. Zoologists have used smear methods effectively in many of their most critical studies for many years, and a real advantage is to be gained by their adaptation to plant cytology.

The adaptation of the smear method to coherent plant tissues seemed unlikely, but the use of strong acids has been found to soften the middle lamella of meristems enough to allow them to be dispersed by pressure. Warmke<sup>16</sup> directs that root tips be killed in 1 part glacial acetic acid and 3 parts of absolute alcohol for twelve hours or more. They are then immersed in 50 per cent hydrochloric acid in 95 per cent alcohol for five to ten minutes, and then transferred to Carnoy's fluid for five minutes. Very small bits of cut-off tip are placed with a drop of acetocarmine solution on a slip, crushed by pressure of a scalpel tip, covered, and gently and carefully heated. Mounts may be sealed, or made permanent by the McClintock method. With sufficient pressure the protoplasts may be much flattened and the long metaphase chromosomes spread out evenly.

*b. Teasing methods* are excellently adapted to many botanical investigations. Much easily disorganized plant material is readily separated into its constituents by patient work with fine dissecting needles. The motion used should be a stroking or combing one, following the direction of the fibers, if of filamentous or fibrous nature. The general operation is similar to that used on animal material (p. 36). It is frequently of advantage to give a brief maceration before attempting to tease material apart. Extremely delicate filamentous algae or fungi are generally teased after partial dehydration and hardening. Rarely is it of advantage to tease material after clearing or infiltration with balsam, but the method may be employed when the products are too minute to be readily retained otherwise (asci of *Peziza*, wood fibers in small quantity).

*c. Maceration of plant epidermis* to facilitate its removal for studies of hair or stomata may sometimes be effected by mechanically stripping the slightly wilted leaf or stem. In most cases, however, the mesophyll (or cortex) tends to adhere and must be loosened before stripping. For the most delicate leaves scalding or boiling may suffice. More resistant cases usually respond to boiling in a solution of KOH (5 per cent, stronger or weaker as results indicate) until the tissues become translucent and tests show that the epidermis comes away most readily. A very careful brushing of the inner surface will generally clear away mesophyll debris. In some cases (*Sarracenia*) the epidermis cannot be

<sup>16</sup> Warmke, H. E. *Stain Technology*, 10: 101, 1935.

stripped, but the epidermis of the partly macerated leaf must be scraped free from subjacent tissue with a scalpel, or the  $\text{HNO}_3\text{-KClO}_3$  method (below) may be adapted with favorable results. After the maceration fluid has been washed out of the material it may be stained with eosin and mounted in 2 per cent acetic acid or glycerin, or mounted in glycerin jelly, or stained with safranin or other suitable dye, dehydrated and cleared for mounting in balsam.

d. *The maceration of woods* to yield wood fibers is an important method because the study of the individual types of tracheal elements in vascular tissues is best effected with isolated fibers. Longitudinal sections permit but a single view of a given fiber and rarely all of it, while suitably macerated fibers show in their full extent. Bundles from an herbaceous fern (such as *Pteris*, which responds very well) or monocotyledons are best separated by hand from the surrounding soft tissue and are cut up into convenient lengths (2 to 10 mm.). Wood from perennial arborescent types (as for instance *Tilia*) is reduced to very thin longitudinal chips or shavings. The material is then gently boiled in 50 per cent  $\text{HNO}_3$  with the frequent addition of crystals of  $\text{KClO}_3$ . It is well to stop treatment when the pieces of material begin to fray out at the ends, but the exact point must be experimentally determined for the particular subject. Wash with several changes of water by settling and decanting, and examine. Boil in safranin O or another strong aniline dye, wash and dehydrate very quickly with 95 per cent alcohol (2 or more changes), absolute alcohol and xylol, allowing the material to settle and decanting the liquid. Nigrosin (saturated) in saturated aqueous picric acid is also a useful stain. While dehydration must be thorough, decolorization is rapid and dehydration must be rushed as much as possible. The addition of a small portion of xylol to the second and subsequent alcohols will reduce the loss of the stain. Material cleared without staining may sometimes be satisfactorily stained in xylol by adding a few drops of a clove oil solution of the dye (as of light green). Maceration of woody tissues may also be effected with very strong hot aqueous chromic acid (30 per cent or stronger). This method is also used to demonstrate the laminated wall structure of *Tribonema* (Heterokontae) and other algae.

## 2. Section Methods.

a. *Grinding.* The preparation of fossil plant material for microscopic study does not offer much variety of method. Something may be learned of the structure of semifossilized peats or of soft brown coals by macerating and bleaching small pieces with Diaphanol, nitric acid, or aqua regia, with subsequent washing and teasing out of the tracheids, spores or pollen grains. Or the samples may be desilicified with hydro-

fluoric acid and sectioned in the sliding microtome with or without imbedding in celloidin under pressure. Jeffery<sup>17</sup> recommends that the material be soaked in carbolic acid under pressure in a wired stoppered bottle in a paraffin oven both before and after the hydrofluoric acid treatment, and the repetition of the treatments with both chemicals, if needed. The periods of immersion in each should be about a week. Staining is not usually practicable, and bleaching (p. 184) is often indicated. Sometimes fragments of tracheids or epidermis can be removed from fossil impressions of leaves or twigs by aid of Diaphanol or nitric acid, but generally microscopical observations are effective only on material infiltrated with silica, etc., without destruction of internal tissues. Harder bituminous coals and fossil woods, seeds, etc., are generally sectioned by grinding. With a hack-saw and a blade of the hardest temper (for cutting steel, etc.) thin slabs should be cut in the desired plane through the specimen. Nuts, seeds and small or irregular pieces of hard material may be set into a soft mass of the so-called wood putty or "plastic wood" which when hardened will grip it firmly for sawing or polishing. For detailed directions regarding grinding sections see p. 353. Radial (and often tangential) slabs can frequently be split from fossil woods with a cold chisel. After the specimen has been ground as thin as possible while held in the hand, one surface is polished. A heavy glass microscope slip or a piece of plate glass is to be ready and clean, and a polished surface of the specimen is cemented to this with the resin-lanolin cement used hot (p. 200). If the cement is too flexible it may be of advantage to reduce the amount of lanolin. As soon as firm and cool the final grinding may begin. After grinding and final washing and drying with alcohol the specimen is to be very carefully slid off the grinding slip, well cleaned with xylol and mounted with thick warm balsam, the cover weighted to squeeze out excess fluid, and allowed to harden. As the specimen approaches desired thinness the danger of its breaking up increases enormously, and great care is required, especially to see that the specimen is of equal thickness throughout, and that the two surfaces are therefore parallel. This is particularly hard with dense black material. Some especially friable specimens have to be saturated with resin before grinding, and cannot be freed from it or the grinding slide before mounting for fear of breaking to pieces. All that can be done is to give a careful washing with water, perhaps a dip through alcohol, finally drying and covering with balsam and a coverglass.

b. *Peeling.* The preparation of fossil plant material<sup>18</sup> has been

<sup>17</sup> Jeffery, E. C. *Anatomy of Woody Plants*. Univ. of Chicago Press, 1917.

<sup>18</sup> Walton, J. *Nature*, 122: 571, 1928.

Graham, R. *Stain Technology*, 8: 65, 1933.



greatly facilitated by a short-cut applied to the grinding method just described. A chosen plane of the specimen is exposed and polished. This surface is etched by 5 per cent hydrochloric acid in water for thirty to sixty seconds. Over the etched surface there is then flowed a liquid composed of 20 second nitrocellulose 20 gm., tech. butyl acetate 200 c.c., methyl phthalate 1 c.c., toluene or xylol 10 to 20 c.c. The films may be allowed to dry from four to forty-eight hours and are then marginally loosened by a sharp blade and peeled carefully off from the specimen to be washed in dilute hydrochloric acid, in water, and then dried between blotting paper sheets under pressure. When dry the "peels" are cleared in Eycleshymer's fluid and mounted in balsam.

*c. Cutting. Free-hand sectioning of plant tissues* gives the opportunity for answering so many of the simpler questions which arise regarding structural conditions in larger plants that all students should be compelled to acquire a considerable facility in the process. For the cruder examinations a safety razor blade held in the fingers or in a flat holder will serve, but for the exercise of skill in producing sections worthy of preservation a regular heavy sectioning razor should be employed. This must be ground absolutely flat (or at most imperceptibly concave) on the back, and honed flat with the imperative avoidance of a secondary bevel near the edge. No "honing back" such as is used on paraffin knives can be tolerated here. The upper face should be ground concave, and held evenly on the hone in sharpening.

The material to be cut is placed between two split pieces of elder pith (p. 178) and held in the left hand supported against the thumb by the forefinger. The razor is held as in shaving by the right hand and rested on the bent forefinger as it is drawn in long sweeping strokes from heel to tip across the specimen. If the arms are resting on the table or against the body there is no likelihood of cutting the supporting thumb even when this is held in the correct fashion much higher than the specimen. The sections obtained will not be as continuous or as uniform as a microtome delivers, but they will reflect in quality the care of the technician, and the speed with which a specimen can be sampled will often more than compensate for a slight loss in quality.

*Hand Microtome Sectioning of Plant Tissues.* Intermediate in convenience between free-hand sectioning and the use of the large sliding microtomes come the hand microtomes.

The essential feature of these is that the knife is not attached to the instrument, but is held in the hand and slid across the glass top of the instrument. It may be a chisel shaped knife or a hand razor, and in the latter case must be held firmly flat on its back upon the instrument. The

specimen is held in a clamp and this is pushed toward the top by a screw at the bottom of the tube, the drum head of the screw being graduated to indicate the degree to which the specimen is raised by the parts of each revolution. An emergency instrument of this type is afforded by a spool of appropriate size, through the hole of which the specimen may be pushed and held by one hand while the razor is drawn across the end with the other.

*Sliding microtome cutting* gives the most exact method of preparing sections of uninfiltreated plant material. While reconnaissance observations of root, stem or leaf anatomy will usually be most quickly and satisfactorily performed upon free-hand sections, the material for final staining and preservation as research data will best be made with an accurate sliding or sledge microtome. Either living or preserved plant material may be sectioned without imbedding provided it is sturdy enough to withstand clamping in the carrier. For the harder woods, seeds, etc., see p. 175. Soft tissues may be imbedded, as in celloidin (p. 180) or paraffin (p. 179), or frozen (p. 178). Stems and roots of ordinary hardness (*Pelargonium*, *Helianthus*) are cut to appropriate lengths and placed between the jaws of the clamp. It is well to protect the material by placing it between split pieces of elder or sunflower pith (p. 178), or even better between pieces of carrot which have been kept fresh and turgid by soaking in water. The knife should be set in as oblique a position as practicable so as to give a long slanting stroke, but for hard tissues the knife may bend too much in this position if it has not a clamp at both ends, in which case only the edge toward the heel can be used. When a knife has been sharpened in a "honing back" it must be tilted to a proportionately greater angle in the holding clamp. Otherwise it will not properly "clear" the specimen in passing over the cut face. The knife and specimen should always be kept flooded with water, or with alcohol of 50 to 70 per cent on preserved or soft material, and the sections removed with the finger or, if great caution is exercised, with a camel's hair brush. Sections may be cut at  $10\mu$  to  $20\mu$  if they are very favorably adapted to the method (*Populus*, *Tilia*) but spongy collenchymatous tissue (*Dieffenbachia*) may have to be cut as thick as  $100\mu$  or more in some cases. Leaves may be cut in bundles, or rolled up, many sections resulting at each stroke of the knife. Very slender roots, moss stems, setae, etc., may be cut in bundles also, and through the later stages handled with a pipette. Softer woods should give no trouble for sections in any plane, but when harder they may have to be boiled for a time to soften them or treated as on p. 162. Generally on this material it is necessary to hone the knife after every few sections

are cut. In this way it may be possible to avoid a wire edge. Hard stems and woods are often difficult to cut if imbedded in paraffin, but if the imbedded specimens are trimmed close to the end and soaked in distilled water for a few days or weeks they are often found to cut very readily. Silicified or carbonized material must be treated with hydrofluoric acid as on p. 161.

*Rotary Microtomes.* These are used for cutting sections of plant material imbedded in paraffin in exactly the same way that they are used for animal material (p. 17). As it is often necessary to sample large quantities of material before deciding which specimens are to be finally completely reduced to sections and preserved, botanists prefer to leave the specimen attached to the block which holds it in the microtome until the test slides have come through the staining processes. This often necessitates a very large number of supporting blocks. These can most economically be prepared by cutting a  $\frac{3}{8}$  inch board of white pine transversely into strips 1 inch wide. These can be quickly split into the desired sizes with a heavy scalpel. One end of each block should be dipped into hot paraffin before mounting any specimens upon it, in order that the pores may become filled with paraffin and the specimen enabled to stick better. One inch lengths cut from dowel rods of appropriate diameter are very convenient.

*Pith for Protecting Plant Tissues.* This is used when sectioning without infiltrating. Pith is usually derived from the elder or sunflower. It is less liable to become soggy when sections are cut if it is soaked and kept until used in an equal mixture of 95 per cent alcohol and glycerin. Since this fluid penetrates slowly it is advantageous to prepare pith for several years in advance of anticipated need. For many purposes fresh root of carrot is preferable.

*The Freezing Method of Imbedding.* This has little general use for plant material. It is, however, important in dealing with fragile pathological specimens and with soft or gelatinous algae. The usual method should be followed (p. 32). Fixed material should generally be brought to formaldehyde (about 4 per cent) and specimens should be coated with egg albumen, gelatin or gum-arabic and attached by it to the carrier. The sections may be handled in syracuse watch glasses or laid upon slips coated with gelatin and cooled. The slides are later warmed to cause the sections to adhere and then treated as in the paraffin method (p. 179), avoiding dyes which might persist in the gelatin.

*The Gelatin Method of Imbedding.* This is frequently of advantage when there is occasion to give special support to fragile plant tissue which is liable to crumble and which becomes brittle in celloidin, such

as diseased bark and woods. Gelatin is allowed to absorb as much water as possible, drained, melted, and the wetted material, cut into as small blocks as practicable, is soaked in this solution. After some hours it is oriented on wooden blocks, the gelatin allowed to cool, and toughened in 10 to 20 per cent formaldehyde. The material should be cut with the knife flooded with water. If the sections are sufficiently strong the gelatin may be removed with hot water, without or with ammonia, and they can then be stained in the usual ways. Material that cannot be parted safely from the gelatin will not stain very satisfactorily, but can be nicely mounted in glycerin jelly if care is exercised not to have the mountant too hot.

*The Soap Method of Imbedding Plant Tissues.* This has been little used, but as advocated by Osterhout for algae, offers advantages applicable to many kinds of mucilaginous or fragile material that cannot safely be dehydrated. He saponifies 70 c.c. of hot cocoanut oil with 38.5 c.c. of 28 per cent aqueous KOH. When firm the product is pulverized. The algae are placed in warm water to which the soap is gradually added until quite concentrated, when by drying it becomes firm enough to attach to a wooden block in the sliding microtome and cut (p. 177). Sections are to be placed on albumen-smeared slides, moistened with xylol and pressed into contact. The soap may then be dissolved away and the slide warmed to coagulate the albumen. Immersion in 95 per cent alcohol should serve the same purpose. The sections may be stained as after paraffin so far as their texture and thickness permit, or the sections may be mounted in water, warmed and examined at once.

*The Paraffin Method of Infiltration and Imbedding.* This process is used in common by botanists and zoologists with only minor differences (p. 6). Plant material, however cleared, is best passed through xylol (2 or more changes) and is then ready for infiltration. This process is subject to the delay caused by the presence of cellulose walls, so that more time is needed than when handling most animal tissue.

If fairly deep dishes (1 cm. or over) are used it is convenient to pour melted paraffin (48° to 56°C.) into them until half full and allow it to harden. The specimens are then placed upon it with enough xylol to cover them. The xylol will gradually dissolve the paraffin and this is complete when the mass has reached a soft pasty consistency. Final infiltration may be effected in either of two ways. The simplest is accomplished by lowering a shaded electric light bulb over a beaker of hardened paraffin until about a centimeter of the wax melts. The specimens are then poured into the melted wax to lie on the solid mass below for several hours until fully infiltrated. The second method involves a well-regulated paraffin oven, where the specimens are placed in small glass

dishes as soon as preliminary infiltration has been accomplished. The dish and its contents should be placed in the paraffin oven at a temperature of about 60° to 62°c. for one-half to one hour, after which the mixture is replaced with melted soft (48° to 56°c.) paraffin at the oven temperature. At the end of one-half hour this may be replaced with a mixture of melted soft and hard (56° to 62°c.) paraffin for another half hour and a second portion of the mixture substituted. This may be left for one-half to one hour at the end of which the material may be imbedded or cast into a hard block. This may be done in thin glass dishes rubbed inside with glycerin, or better in boats folded of good tough paper. The dishes should be chilled and the boats floated in cold water as soon as the specimens have been neatly arranged with warmed needles or forceps.

The data as given are open to modification in several respects. The times of infiltration are to be greatly reduced for filamentous algae, fungi or slender root tips, etc. They are approximately correct for the maximum size of block of phanerogam tissue that should be imbedded for cytological work, i.e., about 2 mm. *They should be reduced whenever possible.* If large blocks of tissue are to be imbedded for histological work the time may have to be greatly increased, but the cell contents will suffer from this exposure to heat. The paraffin should be used of as low a melting point as possible and kept just above that point in the oven. (For a simple and convenient method of keeping paraffin melted see p. 14.) If cutting can be done in a cool room or with a chilled knife a softer paraffin can be substituted with advantage to the material, but if it is necessary to work in the summer an even greater proportion of hard paraffin must be used. The method of substituting paraffin for xylol as given is that which most pleases the present writer. Some workers suggest dropping shavings of paraffin into the vial above the specimens, but this may tend to crush them if delicate. Others suspend a wire gauze basket filled with paraffin in the upper portion of the vial containing the specimens.

*The Collodion or Celloidin Method.* This is used for plants and animals in a very similar fashion (p. 29). The time of immersion must be greatly increased for much plant material, and the sectioning of hard specimens offers considerable difficulty. It is usually best to place the material, saturated with ether-alcohol, in a very dilute celloidin solution, stopper the bottle loosely and allow the solution to concentrate gradually by evaporation. Root tips and highly porous stems will usually be in suitable condition if the concentration is completed in from three or four days to a week, but tissues with strong cellulose walls, or large blocks of wood, should be more closely stoppered and it may take a month or more for them to be properly saturated, for the solution must

concentrate very slowly so as to be of equal density throughout the specimen. If they are sufficiently rigid to stand it (as in woods) the process can be made more rapid by using the solution in 3 strengths of about 2, 4 and 8 per cent. The weakest is first placed on the specimens in a strong bottle and the cork (which must be of the best quality) wired in strongly. Then this container is placed in a paraffin oven at 50° to 60°c. for a day or more as seems to be required. The same process is repeated for the stronger solutions. In any event the material is finally saturated with a barely fluid solution of celloidin. Supporting blocks of fiber or white pine are saturated with ether-alcohol and the end grain dipped in medium strength celloidin solution. They are up-ended and the specimens placed on the soaked surfaces. As soon as the celloidin becomes surface-firm in the air the specimens and the supporting block ends are dipped in strong celloidin, and by alternate drying and dipping a sufficient layer is built up around each of the specimens to support and hold it to the block. Then all are dropped into chloroform until quite firm (twenty-four to forty-eight hours), transferred to 95 per cent alcohol and glycerin (equal parts) until transparent, and may remain there until used. The sections should be cut with a knife flooded with 70 per cent alcohol and placed in a very oblique position. For general remarks on staining see page 573. For plants Heidenhain's hematoxylin followed, if desired, by erythrosin, or safranin O, followed by Delafield's hematoxylin, give the most satisfactory results. Most aniline dyes are unsatisfactory because they stain the celloidin deeply. If this can safely be removed the range of dyes available suffers no special limitations from the imbedding, and ether-alcohol, clove oil and absolute alcohol all will remove the celloidin if the sections are sufficiently tough to remain intact in its absence. Plant material is stained and dehydrated to 95 per cent alcohol in the usual way, and can then be cleared by transfer to carbol-xylol, which the writer prefers to the mixtures of essential oils. After washing with pure xylol the material is mounted in balsam. The celloidin method is generally used with desilicified materials and woods softened with hydrofluoric acid (p. 161). It can readily be so conducted as to permit the consecutive arrangement of serial sections (p. 30). For very hard material advantages are sometimes found in the double infiltration method, whereby a celloidin block is infiltrated with paraffin and cut dry, superior support being claimed for the specimens. It is probable that serious attempts to employ celloidin for cytological studies will produce results superior to the paraffin method in reduction of chromosome clumping or retraction of satellites, etc., but the stages must be most gradual and the sections very thin.

*The Sectioning of Woods and Other Hard Objects Not Adapted to Grinding.* This requires either extremely powerful apparatus or some method of softening the tissues. The study of woods offers two methods of attack, one of more or less macroscopic observation, the other histological. Since distinctions between types of woods can often be made at no or very little magnification, and since these can be facilitated if thin slices be used, it has become customary to prepare large sections and to use these as hand specimens<sup>19</sup> or if sufficiently thin to use them for lantern projection. While special machines for cutting these slices have been made, they are rarely available. Remarkably good work can be done with a heavy steel carpenter's plane provided the blade is of hard temper and skillfully sharpened to a keen straight edge. Transverse sections made with this may be 1 to 2 inches broad and up to 6 to 8 inches long, and if cut from radial slabs from a tree may show cork, phloem, xylem and pith in fine condition. The sections can be cut thin enough for low power microprojection (5 to 10 cm. focal length lens). While such woods as pine and cedar cut most easily, *Wistaria*, *Vitis*, *Acer*, *Pyrus*, *Castanea* and even *Quercus* are perfectly practicable. The larger sections and harder woods make much the greatest demands on the skill of the cutter. Sections should be kept wet and may be stained (safranin, Heidenhain's hematoxylin, etc.) in petri dishes or other suitable containers, dehydrated and mounted between thin sheets of glass such as lantern slide covers. The mounting balsam should be very thick, used preferably quite warm, and the mounts should be kept warm (as in a paraffin oven) until quite hard. This is especially urgent if the mounts are to be exposed to the heat of a lantern. It assists in mounting to have one of the mounting glasses a little smaller (5 mm.) than the other, and it may be necessary to use a weight to hold the specimen flat if it is not very thin.

For accurate microscopical study of cell types and arrangements this method will not suffice. Soft woods or the alburnum of harder ones, especially in fresh material, can frequently be cut in a sturdy microtome without special preparation (p. 177). Seeds that are hard because of the presence of reserve cellulose can likewise best be cut fresh before they have become entirely mature to dryness. If dry they may become soft enough after boiling in water. Sections cut deliberately thin are more easily obtained than thicker ones. Very hard seeds may be treated with hydrofluoric acid or after the fashion of fossil woods (p. 174). It has recently been suggested that the direction of steam on the surface of the block as it is being cut would so soften the wood at the surface as to render it easily divided by the knife. This<sup>20</sup> is effected by boiling water

<sup>19</sup> Hough, R. B. *The American Woods*, Ed. 2, N. Y., 1893-1913.

<sup>20</sup> Crowell, I. H. *Stain Technology*, 5: 149, 1930.

in a flask and conducting the steam to the block through a metal tube. The sections can be cut as thin as  $10\mu$ , even from hard woods and without the usual degree of dulling of the knife or the chemical changes induced by hydrofluoric acid. Hard woods may be softened as much as possible and imbedded. As the hardness is often due in large part to the deposit of mineral matter (especially silicates) in addition to lignification it is best to remove the mineral matter before imbedding. This is effected through immersion in hydrofluoric acid which has little effect on the middle lamella and even leaves the coarser cell contents intact. Dry woods should be very thoroughly freed from air by alternate open boiling and exhaustion under an air pump, and in fact this is usually necessary with fresh woods. Pieces as small as the requirements permit (preferably 1 cm. or under) are placed in full strength hydrofluoric acid in a wax or wax-coated container and allowed to soak for one to several weeks, in the latter case changing the solution occasionally. Comparatively soft woods may be given the reduced period, or even soaked in once used or diluted acid. After the maximum softening has been attained, as determined by testing the blocks with a knife, the acid must be very thoroughly washed out. Imbedding is usually in celloidin, and if the material is porous and homogeneous by the rapid method (p. 181). The knife used on woody material should be particularly heavy and rigid, should preferably be clamped at both ends, and may be placed obliquely or in a more transverse position if that gives better results. It will generally need honing after every few sections, or in extreme cases every section, to avoid a turned or wire edge. The sections generally will not withstand removal of the celloidin and must be stained accordingly.

*d. Affixing Methods.* When handling paraffin sections, or celloidin sections in serial order, it is usually necessary to attach the sections to microscopical slips and by changing the glass with its attached sections to perform the remaining manipulations with a minimum of effort.

For paraffin sections it generally suffices to rub an exceedingly thin film of Mayer's albumen (p. 620) on the slip, following with a half-pipette full of distilled water on which the sections are floated and spread by gentle heat, the excess water being then drained off and the sections accurately arranged. It is generally necessary to maintain the sequence of the sections through the entire series, mounted on a slip, and, in order that a mechanical stage may be effectively used in recording observations, to keep the rows straight. Szombathy's affixing method involves the flooding of the slip with a solution consisting of gelatin 1 gm., water 100 c.c., sodium salicylate 0.02 gm., glycerin 15 c.c., to which there is immediately added a few drops of 2 per cent formaldehyde solu-



tion. The subsequent procedure is the same as for Mayer's albumen formula, and the result is usually a cleaner slide with firmer attachment of the sections. For sections that tend to expand and contract considerably during dehydration and hydration it may be advantageous to use a mixture containing gum arabic or glue 1 gm., potassium bichromate 1 gm., water 98 c.c. (Land). The slide may be flooded with this, the sections spread and the slide drained. Exposure to daylight will render the gum insoluble in the ordinary histological reagents. The use of a dilute solution of albumen instead of rubbing the slip with a minimum quantity of the strong preparation has not as wide acceptance among botanists as among zoologists, but it will work with such cytological material as has densely protoplasm-filled cells.

For celloidin sections the methods used by zoologists (p. 31) are adequate.

### III. Preparation and Staining of Sections

*A general preparatory treatment* is required of most sections of plant material previous to staining. Separate paragraphs have been prepared dealing with problems specially related to individual major groups of plants and detailed information as to methods applicable to them should also be sought there. Others have been prepared treating of the various imbedding, sectioning, macerating, staining and mounting methods, as well as methods pertinent to special tissues. Sections cut free-hand or with the sliding microtome need little treatment preliminary to staining. If they are highly mucilaginous it is generally necessary to remove this substance before staining by boiling in a relatively large volume of water (or several changes) until the mucilage has been completely dispersed. Otherwise it will be difficult to differentiate the stains, to prevent the sections from curling and to dehydrate them properly. Starch is frequently objectionable as obscuring cell arrangement, and can be removed by prolonged boiling of the sections in water. As the starch paste formed at first in the cells is as bad or worse than the grains the washing should be complete and the sections should be tested with aqueous iodine until the starch reaction is negative. It may be necessary to add a few drops of hydrochloric acid to the water to facilitate removal of the starch. A somewhat prolonged treatment of the sections with diastase may also be used to remove starch. Chlorophyll may be objectionably prominent in chloroplasts and although it usually disappears in the dehydration it may be better to soak the sections for a time in 95 per cent alcohol before staining.

*Bleaching* is necessary if plant tissues are naturally colored, or de-

velop pigments in the cells as a result of the method of preservation, before stains may be applied. Discoloration due to osmic acid is readily removed from sections in commercial hydrogen peroxide diluted three-fourths with water, or one-half with 95 per cent alcohol, which latter solution usually keeps for several days. The bleaching is hastened by exposure of the material to sunlight. Free chlorine may also be used. A few drops of hydrochloric acid are dropped on a crystal of potassium chlorate, and when gas is formed 50 per cent alcohol is poured on it. The specimens are placed in this alcohol from 70 per cent and bleach in one-fourth hour or longer. The method can be used with sections attached to a slip. Immersion in freshly prepared Javelle water is often very effective. Sulphurous acid saturated in alcohol may be used to bleach, or potassium permanganate in very dilute solution followed by oxalic acid and exposure to light. Leaves (especially *Drosera*) which blacken when thrown into alcohol are advantageously immersed when collected in a rather strong NaOH solution at room temperature until they become bleached and translucent. After washing they may be preserved in alcohol and remain translucent and undarkened.

*Staining methods for plants* have been developed along several lines, of which the more general are outlined here. Staining of plants in toto has been almost discarded for the grosser types, although for unicells and for filamentous types it still serves a good purpose. For details see paragraphs on algae, bacteria, fungi, etc. (p. 226). Staining of vascular systems in pieces of stem or leaf to trace the course of the bundles may be accomplished with the living plant, killing it afterward (p. 212).

Staining methods for histological or for cytological features employ solutions prepared in similar fashion. The use in botanical practice of mixtures of dyes, especially such as involve chemical action and unstable compounds in the solution, has largely passed away. This enables a few general directions to be given for the preparation of standard stock solutions. Special staining methods will be considered separately. (For a more complete discussion of the properties of biological dyes see p. 573.)

Aqueous dyes are usually made up as 1 to 2 per cent solutions. Since the proportion of dye substance in the powder is not constant it would be better to prepare them as saturated solutions, which probably would not affect the practice greatly, as most of the dyes are soluble to approximately 1 to 2 per cent of actual substance. Among the common dyes frequently used in aqueous solutions are: aniline blue (for sieve tubes), methylene blue, gentian and crystal violets, eosin, carmine, hematoxylin and picric acid. Distilled water should always be used. The anilines all function well as 1 per cent solutions. The aqueous hematoxylin is the simple solution for use in the various separate-mordant methods. The

dyestuff should be of pale brown crystals and of assured staining quality, for many samples differentiate poorly. The stock solution is of 1 per cent strength, and in use it is diluted to  $\frac{1}{2}$  to  $\frac{1}{4}$  per cent (histology, most cytology) or  $\frac{1}{10}$  per cent to  $\frac{1}{20}$  per cent (filamentous algae, etc.). The stock may be prepared by gradual solution in the cold, or by the aid of heat, but the latter had best be avoided for critical work, and preliminary solution of the hematoxylin in alcohol must absolutely be omitted, for the resulting solution behaves in a quite inferior fashion.

Alcoholic solutions are, like the aqueous ones, generally 1 per cent strength. Saturated solutions would be more constant in composition, but where used for counterstain should be somewhat diluted before use. Common dyes are generally made up in 70 per cent alcohol and include the following: acid fuchsin, light green SF yellowish, fast green FCF, bismarck brown, crystal and gentian violets (for histological work), alcoholic eosin and erythrosin, auramine, phloxine B. The writer uses "aniline water" (a saturated aqueous solution of aniline oil, prepared by shaking and filtering) in making up the gentian and crystal violet solutions, but not the others. Solutions with aniline oil do not always keep as well as without it, but they generally stain more densely. Aniline blue, when used for filamentous or unicellular organisms that are inconvenient to dehydrate after staining, is usually made up in 90 per cent alcohol. Safranin O, the most commonly used aniline in histological researches, is best prepared as a 1 per cent solution in 50 per cent alcohol made with aniline water (see above). If the color tone of either the safranin or the light green solutions is too cold to be pleasing the writer adds about one-fourth per cent of auramine to the solutions, effecting a considerable improvement in the preparations.

Delafield's hematoxylin combines mordant and dyestuff, affording a very valuable histological stain. Other combinations of different mordants with hematoxylin have been proposed, but in plant histology this one answers all requirements, and is most regular in action.

It is often desired to apply to plant material a stain which will act without the previous interposition of fixing and mordanting processes. The dye used is usually a toxic one and the cells do not survive the treatment. Material such as plant epidermi, *Spirogyra*, etc., may well be stained in about five minutes by a 1 per cent aqueous solution of eosin which is differentiated with water and fixed with 2 per cent acetic acid (p. 198). If desired the material may first be treated with aqueous iodine-potassium iodide solution to stain the starch, and after rinsing, the eosin may be applied. Another excellent stain is picro-nigrosin, the latter dye being dissolved to the desired degree (as, for instance, 1 per cent) in saturated aqueous picric acid. The material can be washed, dehydrated and

mounted in balsam. The acetic acid-methyl green mixture used on protozoa also serves well for many algae.

Single stains are often used in plant histology and cytology when it is desired to bring out one kind of structure clearly without much reference to the associated structures. Frequently these hold a little of the stain used, of course, and are not invisible by any means. Heidenhain's hematoxylin is used in this fashion for cytological work, and safranin O for both cytological and histological work. Almost any of the primary dyes to be considered below can be used in this way if the counterstain offers no advantage. The differentiation is carried somewhat further than if a counterstain is to follow.

Many staining methods are closely bound in their effectiveness to the pH at which they are conducted. Many suggestions point to the development of a rational staining system based on specific acidity and buffer action. As yet for most plant processes it is well to adhere to the older empirical system in routine work, but all solutions must be made up with distilled water. Further, all procedures involving a stage of alkalization usually expressed as "washing in tap water" should be revised to specify washing in distilled water made alkaline by the addition of a designated small quantity of a known substance. Naylor<sup>21</sup> has shown clearly how the controlled pH of solutions can affect the staining effect on plant protoplasm. Buffer solutions for use with stains have been formulated.<sup>22</sup>

Combinations suited for ordinary histological work are as follows:

*Safranin O followed by Delafield's Hematoxylin.* Soak the sections in safranin three to twenty-four hours, or boil them in it if they will stand it. Destain with 50 per cent alcohol (acidified with acetic acid should the stain come away too slowly) until the stain ceases to come off freely and the soft tissues are simply pink against the red lignified ones. Wash in distilled water. Counterstain in diluted (about 10 per cent to 20 per cent) Delafield's hematoxylin until sufficiently dark, wash in several changes of tap water or in distilled water containing a trace of  $\text{NH}_4\text{OH}$ , then dehydrate, clear and mount in balsam. Lignified tissues, nuclei, cuticle and cork stain red; cellulose, collenchyma and chromatophores purple. Poor contrast is generally due to insufficient washing of the hematoxylin, for by washing the originally reddish stain becomes blue-purple. This stain is the most permanent of the histological combinations (unless perhaps the next) and should be depended on for research record purposes.

<sup>21</sup> Naylor, E. E. *Amer. J. Bot.*, 13: 265, 1926.

<sup>22</sup> French, R. W. *Stain Technology*, 5: 87, 1930; 7: 107, 1932.

*Heidenhain's Hematoxylin and Eosin (or Erythrosin).* Mordant sections one to three hours in 1 per cent iron-ammonia-alum solution. Wash carefully in distilled water for five minutes. Stain in  $\frac{1}{10}$  per cent hematoxylin until sufficiently dark, or in  $\frac{1}{2}$  per cent hematoxylin for one to three hours, rinse and destain to the desired degree in iron-alum solution. Wash fifteen minutes to one hour in tap water, dehydrate to 70 per cent alcohol, counterstain with alcoholic eosin or erythrosin, complete dehydration, clear and mount. Depending on the amount of differentiation, the hematoxylin is limited to the middle lamella of the xylem tissue or it is completely stained. Lignified tissues and nuclei stain black, cellulose and all softer elements pink. This combination is satisfactorily permanent, and more capable of precise differentiation when studying the structure of woody tissues alone, than the former, but more trouble and less generally useful.

*Safranin O and Light Green.* Soak the sections in safranin three to twenty-four hours or boil them if they will stand it. Destain with 50 per cent alcohol (acidified with acetic acid should the stain come away too slowly) until the stain ceases to come away in clouds and the parenchyma is pink rather than red. Transfer to alcoholic light green for one to ten minutes, rinse in 70 per cent alcohol, differentiate with 95 per cent alcohol, clear and mount. If the stains come away too readily, pass from light green to absolute alcohol and differentiate there. If they hold well, use more intermediate changes of alcohol so as to cause as little shrinkage as possible. Lignified tissues, cuticle and cork red, cellulose and collenchyma green. The stain is a splendid one, of brilliant contrast, and easily mastered. Not suited for tissues with little lignification. It is sufficiently permanent for ordinary class purposes, and to be preferred to the following.

*Safranin O and Fast Green FCF.* This combination is handled much as the foregoing. The green is of a somewhat more bluish tone but is regarded as much more permanent. Moore<sup>23</sup> has suggested a modified schedule which offers some advantages. The safranin stock consists of a 1 per cent aqueous solution. The fast green stock is a 0.5 per cent solution in absolute alcohol. After paraffin has been removed from the affixed sections the slides are hydrated to 70 per cent alcohol and are plunged into staining jars in which water has been colored with 20 drops of the stock safranin. They should stain in one to twelve hours, and then are rinsed successively in 70, 95 and absolute alcohol. Differentiation is accomplished in a well of the fast green solution, the slides being kept in motion, the excess green being washed out with absolute alcohol and the slide cleared and mounted in balsam.

<sup>23</sup> Moore, J. A. *Stain Technology*, 11: 69, 1936.

*Safranin O and Crystal (or Gentian) Violet.* Conducted as safranin-light green. Cellulose and collenchyma violet. Offers no advantages, and the violet fades more readily than light green or Delafield's hematoxylin, and offers less contrast than the former.

*Safranin and Aniline Blue.* This follows the general schedule for safranin and light green, but after a very brief differentiation in 95 per cent alcohol the aniline blue should be fixed and intensified by substituting a slightly acidulated (HCl) alcohol. A strong stain in safranin is necessary. After the acid treatment the sections are washed with neutral 95 per cent alcohol, dehydrated, cleared and mounted. Cellulose walls and collenchyma brilliant blue. A good contrast stain when well carried out, but somewhat more tricky than safranin-light green and not so good for photographic purposes.

*Safranin O and Orange G.* Handled like safranin and light green. A very fine stain to use for photographic purposes when strong contrast is needed between all tissues and the background without losing details within the tissues. Cellulose and collenchyma orange.

*Auramine and Aniline Blue.* Handled like safranin and aniline blue. More suitable for photographic work, as the contrast is not so severe nor detail in the xylem so hard to secure. It is somewhat more difficult to retain the auramine in the xylem than the safranin. Lignified tissues, cutin and cork, bright strong yellow; cellulose and collenchyma bright blue.

*Iodine Green and Acid Fuchsin.* Soak for several hours in iodine green, or boil in it if the sections will stand it. Destain very briefly with 95 per cent to 100 per cent alcohol. Counterstain with acid fuchsin (often best diluted with 1 to 5 parts of 70 per cent alcohol) for two to three minutes. Rinse and differentiate with absolute alcohol. The difficulty lies in losing too much of the green. Lignified tissues, cuticle and cork green; cellulose and collenchyma pink. The stain fades rather readily, but lasts two to five years if protected from light. It is invaluable in providing a stain with the colors reversed from the usual safranin-light green to use where the xylem is too dense to photograph well when stained red and to dispel the idea afflicting classes that xylem is necessarily red and parenchyma green.

*Bismarck Brown and Light Green.* Stain lightly with bismarck brown, destain with 50 per cent alcohol, counterstain with light green for one to five minutes, differentiate with 70 per cent alcohol, dehydrate, clear and mount. Lignified tissues, cuticle and cork brown; cellulose and collenchyma green. Not a stain capable of sharp differentiation, but satisfactorily permanent and excellently suited to photographic work.

*Crystal (or Gentian) Violet and Orange G.* Handled like the safranin-light green. A brilliant stain when successful, but somewhat erratic. The gentian violet will come away rather readily. Lignified tissues, cuticle and cork violet; cellulose and collenchyma orange.

In cases where differentiation with an aqueous or alcoholic counterstain cannot be accomplished because of too great loss of stain from the tissues, the material may be dehydrated to 95 per cent alcohol, differentiating the primary stain as well as possible. Then it is cleared with a solution of the counterstain dissolved in clove oil. Suitable dyes are crystal or gentian violet, erythrosin, light green and orange G. If these dyes go directly into solution too slowly, they may be moistened with absolute alcohol or about 20 per cent of this added to the clove oil. The stock solutions should hold about 1 per cent of the dye, but this may have to be diluted, especially with the gentian violet. This method of staining seems to simply "paint" a coat of the dye on the unlignified or other unstained tissues, and while it is often very brilliant it cannot be trusted too closely as to differentiation. See remarks under cytological methods (p. 220).

Combinations suited to ordinary cytological work are as follows :

*Heidenhain's Hematoxylin Method.* This is the finest stain available for cytological work. It is adaptable to most structures, exceeds all others in precision on chromatin-containing elements and many other parts, and in general dependability and permanence far outdistances all others. No student of plant cytology can afford to omit a thorough training in the use of this stain and in the interpretation of the results it produces. (For directions see pp. 609, 613.)

*Tuan Modification of the Hematoxylin Stain.* It has been found possible to secure increased sharpness of the hematoxylin stain by careful modification of the usual sequences as shown by Kaufmann (p. 170) and others. A very significant improvement has resulted from the employment of picric acid in place of iron-alum as reported by Tuan.<sup>24</sup> The schedule is a flexible one and may profitably be modified, as he has shown, for various materials. A general form is as follows: Material fixed in a chrom-osmo-acetic mixture is best. After having been bleached in  $H_2O_2$  (p. 184), wash again, stain in 0.5 per cent aqueous hematoxylin for twenty minutes, wash ten minutes, destain in saturated aqueous picric acid as determined by a test slide, and wash again with a trace of ammonia in the water. The time required will vary from twenty minutes to two hours or more, and should yield quite colorless cytoplasm and black chromatin-bearing structures. Since the dye is decolorized while in the picric acid it is necessary to wash the test slide from time to time

<sup>24</sup> Tuan, H.-C. *Stain Technology*, 5: 135, 1930.

to restore the color and judge the degree of destaining. It is necessary that the mordanting and staining be limited to short periods, in order that the differentiation be not unduly prolonged.

Following Heidenhain's hematoxylin one may use orange G, erythrosin or light green as counterstains, either in alcohol or in oil, but rarely to advantage. The process calls for one to twelve hours mordanting in the iron-alum, a thorough washing for five to fifteen minutes in changing water, one to twelve hours in hematoxylin, then rinsing, differentiation in the original iron-alum solution or a weaker one if better control is needed, a thorough washing for fifteen minutes to one hour in changing water, slow to gradual dehydrating and clearing and finally mounting in balsam. Differentiation should be completed under observation. If the approximate time is determined by a trial the bulk of the slides can be carried through the destaining in wells to within a very few minutes of the correct point. Then the iron-alum can be replaced by clear water and the slides removed in pairs and placed face upward in a Petri dish of iron-alum on the stage of a compound microscope where the process can be completed under direct control with the 16 mm. or 8 mm. objectives, following with the usual washing.

*Brazilin* has been used as a cytoplasmic stain by Belling, Webber<sup>25</sup> and others. While it is doubtful if it offers any advantages over a properly conducted hematoxylin procedure, the fact that the water stages of the latter may be avoided may be of importance. Smears are fixed in a mixture of 10 c.c. of glacial acetic acid and 25 c.c. of absolute alcohol for ten minutes, washed in absolute alcohol thoroughly and hydrated to 60 per cent alcohol. Mordanting is effected in one hour in a mixture of 20 c.c. of absolute alcohol with 15 c.c. of 4 per cent aqueous iron-ammonia-alum. The excess is washed out with 60 per cent alcohol and three changes of eight minutes' duration in 70 per cent alcohol. The smears are then stained in 0.5 per cent brazilin solution in 70 per cent alcohol for an hour, dehydrated through 70, 80, 95 per cent and absolute alcohols in five-minute changes, then dropped to 70 per cent alcohol to destain the chromatin to the chosen degree. The cytoplasm is then cleared in the mordanting mixture, the slides rinsed well in 70 and 95 per cent alcohols, dehydrated in two changes of absolute alcohol for five minutes each, cleared in clove oil for fifteen minutes, passed through 80 per cent xylol with clove oil to pure xylol and mounted in balsam. The rough treatment involved in the fixing and dehydrating stages render this method inherently untrustworthy for fine work.

*Safranin O-Crystal (or Gentian) Violet.* This stain is more readily accomplished than the next, but not capable of such exact and beautiful

<sup>25</sup> Webber, J. M. *Univ. Calif. Publ. Bot.*, 14: 345, 1929.



differentiation. Stain sections in safranin for three to twenty-four hours, destain for five to thirty seconds or more in 50 per cent alcohol, counter-stain in aqueous gentian violet for ten seconds to a few minutes, partly differentiate and dehydrate rapidly with 50, 70 or 95 per cent alcohols, clear and complete differentiation in clove oil. This may take some time, an hour or more. If too much stain is lost with this alcohol series reduce it to the 95 per cent alone, or even replace that by 100 per cent. Wash through 2 or 3 changes of xylol and mount in balsam. The length of time that the safranin should be allowed to destain will be found very variable and a critical feature, and will depend on the time the sections are left in crystal violet as well as the character of the material and of the safranin. It may be necessary to acidify the alcohol. Nucleoli and chromosomes stain bright red; resting nuclei (except nucleoli and heavy chromatin granules) deep violet; prophase and late anaphase nuclei with purple spireme show chromatin granules red, if present; cytoplasm very light violet; spindle darker violet. The stain does very well after chrom-acetic mixtures, where there is little advantage in the following triple stain. It is possible to substitute crystal violet in clove oil for the aqueous solution, giving a more brilliant violet but with little differentiation beyond the nucleoli and chromosomes. It should be used quite dilute (1: 10) and any deposit of crystal violet removed with clear clove oil. This method and the next are rendered unsuited to very critical work on the structure of the spireme and of the chromosomes by reason of the violent changes in alcohol concentration made necessary by the rapidity with which the stains are extracted from the paraffin sections.

*Safranin O-Crystal (or Gentian) Violet-Orange G. (Flemming's Triple Stain).* This beautiful combination has had a great vogue. Undoubtedly very effective when correctly done, it is difficult to master. The violence of the alcohol changes necessary are quite out of the spirit of modern cytological methods, which strive to avoid any changes likely to disturb the cell structure, and the method is not suited to studies on the structure of the spireme or of the chromosomes or of any other cell organs easily disturbed by the convection currents or shrinkage. It is not possible to give any precise schedule for this any more than for the previous method. Its adaptability over a wide range of material is very great, but for any given piece of material it requires a very exact schedule: its flexibility under given conditions is very low. It is not a suitable stain to use after Bouin's solution or mercury mixtures unless the sections have been treated with chrom-acetic fixing solution (or once-used Flemming's fluid) for twenty-four to forty-eight hours properly to mordant them. It is peculiarly adapted to chrom-osmic-acetic fixed material. Sections should be stained in safranin for six to twenty-four hours. They are

then destained in 50 per cent alcohol until safranin no longer comes away freely, but remains in the nucleoli and chromosomes. If even these lose the stain the material is not suited to this method. The sections are then immersed in aqueous crystal violet for thirty seconds to ten minutes. The violet should have time to replace the safranin in all except the chromatin-containing structures. The sections are then rinsed in water and immersed in aqueous orange G to differentiate the violet. The time should be short; fifteen to sixty seconds perhaps. Then the slides must be rapidly dehydrated, differentiated and cleared in clove oil. The dehydration must frequently be accomplished with one to two changes of 95 per cent and 100 per cent alcohol, that the stain be not lost. The ultimate product should show red nucleoli, chromosomes and chromatin granules, violet spindle fibers and plastids, violet linin in the nucleus and pale buff-gray cytoplasm. Orange as such will only appear slightly, and in the cell walls. Its primary function is to sharpen the differentiation of the other two dyes. The general effect is not as gaudy as the preceding combination, but the precision of detail should be much greater. The use of oil solutions of crystal violet and orange G is to be avoided; the result is not so accurate nor is the method in the spirit of the original combination.

*Cyanin and Erythrosin.* The stains should be in 70 per cent alcohol. Stain the sections in cyanin for a few minutes, or if the stain comes away too readily extend the time to one or several hours. Rinse the sections in 70 per cent alcohol very quickly and stain for a few seconds in erythrosin. Then 95 per cent and 100 per cent alcohol. Clear in clove oil, to xylol, and mount in balsam. If the stains come away too readily omit the 95 per cent alcohol and the clove oil. Chromosomes blue; cytoplasm pink. As a histological stain, lignified structures blue, cellulose pink.

*The Feulgen Reaction* has been slowly gaining favor in cytological studies on plants, particularly where the cells under observation contain considerable material which would stain by the hematoxylin or safranin techniques. It is not as uniformly successful as appears to be the case in animal studies. The schedule proposed by Margolena<sup>26</sup> may be taken as representative.

"Fix preferably in saturated  $\text{HgCl}_2$  with 2 per cent acetic acid; or other fixatives may be used, as will be mentioned later. Wash thoroughly, varying the time with the size of the object. Run up through the alcohols, imbed, section and mount. Run sections down to water and treat with 0.5 normal HCl for two or more minutes, treat with the same at 60°C. for two or more minutes, rinse in the cold HCl, then in water and stain in the fuchsin sulphurous acid for one to three hours. Prepare this as follows: dissolve 1 g. basic fuchsin by bringing it to boiling

<sup>26</sup> Margolena, L. A. *Stain Technology*, 7: 9, 1932.

in 200 c.c. distilled water, cool to 50°C., filter, add 20 c.c. of the 0.5 normal HCl, cool to 25°C. and add 1 g. sodium bisulfite. Cork well and put in a dark place. The solution should decolorize to a straw color, usually within twenty-four hours. After staining pass through three successive baths of 200 c.c. distilled water with 10 c.c. of 0.5 normal HCl and 10 c.c. of 10 per cent sodium bisulfite. Rinse in distilled water, run up through the alcohols, counterstain, if desired, with any of the usual counterstains, dehydrate, clear and mount. The kind of fuchsin used for Feulgen's reagent is of great importance, since some fail to decolorize, give a non-specific brown reaction, or fail to react at all. Fuchsins of the rosanilin type seem to be best adapted for the purpose."\*

*Latex and latex vessels* in plants are preserved by methods already given (p. 159). Sections are generally cut longitudinally. Stained in a dilute aqueous iodine solution followed by aqueous eosin or erythrosin, they should show the tubes as rose-pink structures containing rich purple rounded or femur-shaped starch grains (as *Euphorbia tirucalli* or *E. splendens*), and may be mounted in 2 per cent acetic acid or dehydrated, cleared and mounted in balsam, but the stain of the starch cannot be made permanent. If the sections (*Ficus elastica*, etc.) are treated with absolute alcohol various soluble substances are removed and the caoutchouc left. Latex vessels (*Tragapogon*) are best shown by a rather heavy stain of safranin, subsequently dehydrated, cleared and mounted in balsam. Latex cells (*Sanguinaria*) also are best treated in this way.

*Sieve Tubes.* Material should be preserved as indicated on p. 159. Sections cut transversely will show the faces of the plates, cut longitudinally, the relation of the contents to the pores, but in the average plant they are not easily shown. For demonstration to classes Curbitaceae give suitable material. *Cucurbita Pepo* should be stained for twelve to forty-eight hours in a 0.001 aqueous solution of aniline blue, rinsed and dehydrated to 70 per cent alcohol, counterstained with 1 per cent eosin or erythrosin in 70 per cent alcohol, the dehydration completed and the sections cleared and mounted in balsam. This progressive stain should need no differentiation, but if desired the sections may be stained in a strong solution for a few hours and differentiated in glycerin. Stains with aniline blue can be fixed and somewhat strengthened by treatment with a mild alkaline solution ( $\text{NH}_4\text{OH}$  or  $\text{Na}_2\text{CO}_3$ ). The callose of the sieve plate should be blue, as also the lignified tissues, and the rest of the preparation pink.

*Sperms in plants* if abundant are in general most readily fixed in a drop of water on a slide, inverted over a bottle containing osmic acid either solid or in solution. The sperms are quickly and nicely fixed by

\* After this account was in type, a promising revision of the Feulgen technique appeared by J. A. de Tomasi: Improving the technic of the Feulgen stain. *Stain Technology*, 11: 137-144, 1936.

the vapor, which dissolves in the water in which they are suspended. These slides can then be dried and stained with iodine green and acid fuchsin, Delafield's or Heidenhain's hematoxylin, Flemming's triple stain, etc. If the cilia show with difficulty, Loeffler's flagellar stain (p. 142) or carbol-fuchsin (p. 142) may be tried. The large sperms of Cycads are best fixed in the enlarged ends of the pollen tubes before they are shed, and sectioned (p. 242).

#### IV. Microchemical Reactions in Plant Membranes

Quite apart from the use of microchemical methods to determine the nature of the walls of plant cells is their use to differentiate tissues from each other for purely histological purposes. The reactions involved rarely give permanent preparations. For the more specialized procedures the section on microchemistry should be consulted.

*Cellulose* is probably the wall material most commonly met with among higher plants. It is frequently impregnated with other substances. A good reaction is generally secured if sections are soaked in a solution of 0.3 per cent iodine and 1.3 per cent potassium iodide in water, following this with a few drops of 60 to 70 per cent sulphuric acid. The cellulose turns blue, the lignified tissue becomes somewhat more yellow than before.

*Chlor-zinc-iodine*<sup>27</sup> is used as a test for celluloses in the cell walls of plants, especially fungi and algae. "Dissolve 20 gm. of  $\text{ZnCl}_2$  in 8.5 c.c. of water with the aid of heat. Cool and add, drop by drop, the following solution until, after shaking, the iodine which is precipitated fails to dissolve further: 3 gm. of KI, 1.5 gm. of  $\text{I}_2$ , and 60 c.c. of water. About 1.5 c.c. of the latter solution is necessary." The mixture must be made exactly as specified and should be kept in a glass-stoppered dropping bottle. Small samples of material should first be treated with the  $\text{I}_2$ -KI solution, this drained from the slide and replaced with the chlor-zinc-iodine under the coverglass; treatment may be repeated to advantage in accentuating the reaction. The walls which contain cellulose show a blue coloration varying in intensity and tone, according to the amount and character of the cellulose material present.

*Lignin* is one of the commonest tissue-modifying substances replacing cellulose. A solution of phloroglucin followed by 50 per cent hydrochloric acid gives a splendid red purple coloration of such tissues. Aniline sulphate (saturated aqueous) followed by sulphuric acid gives a good golden yellow color to lignified tissues.

<sup>27</sup> Nowopokrowsky; cited by Drouet, F., *Bot. Gaz.*, 55: 695, 1934.

*Cutin* is generally deposited on the outer wall of the epidermal cells of aerial parts of plants. In the case of xerophytic plants it may become quite thick, as on the leaves of *Aloë*, *Gasteria*, or of *Hedera*. It stains with various substances, such as chlorophyll, alkanin and sudan III, and in ordinary histological practice with those that also stain cork and wood.

*Suberin* is the typical waterproofing substance in cork, and appears elsewhere in plant tissues. When present the tissues become comparatively insoluble in sulphuric and chromic acids, becoming invisible in the latter because of its dark color, but dissolving only after some days of exposure. Suberin gives the staining reactions described above for cutin. Neutral violet (1:10,000 aqueous, slightly acidified) is reported to stain cork violet, but not to stain cuticle. Like cutin, cork turns yellow-brown after chlor-zinc-iodide in sections which have been treated with javelle water and washed with 1 per cent hydrochloric acid, while cellulose turns violet.

*Pectin* appears principally as a calcium salt in the cementing substance in tissues of higher plants, joining the cells together. It readily dissolves in macerating fluids (p. 174), or, in fruits, even in simple boiling water or very dilute acids. It stains brown-red with neutral violet (above), and also with ruthenium red, which also stains gelatinous derivatives.

*Mucilage and gelatinous walls* are readily stained by methylene blue and gentian violet. (See the method designed by the writer for algae p. 228). "Slimes" may to some extent be differentiated by their color reactions, but in nature are generally mixtures. Cellulose-derivatives hardly react to iodine and stain with congo red in an alkaline solution. Pectose-derivatives are coagulable with lead-acetate and with alum, and become brown with iodine. They stain with hematoxylin, methylene blue, neutral red and ruthenium red in neutral solutions. Callose-derivatives dissolve with dilute sodium and potassium hydroxides very readily, and color with aniline blue in an acid solution.

*Resins* in plant tissues are often stained green by copper-containing preserving fluids. They may be removed by mixtures of xylol and absolute alcohol.

*Callose* generally appears in the sieve plates of phloem tissue, and also in pollen grains and tubes, and the mycelium of fungi. It is very strongly colored by a dilute aqueous solution of corallin in 4 per cent soda, or by dilute aqueous aniline blue followed by dilute hydrochloric acid.

*Silica* can usually be recognized after incineration of the sections, the siliceous portions remaining comparatively unaltered. The silica can be removed with hydrofluoric acid (p. 161).

## V. Mounting Methods

1. The refractive index of the mounting medium chosen is of considerable importance to the botanist because so much material is studied unstained. If it is desired to exhibit the structure of delicate colorless parts a medium with a refractive index either much higher or much lower than the specimen should be chosen. On the other hand, if the object is dense and it is desired to make it more transparent a fluid of equal density should be chosen. Of course the nature of the specimen must be considered: sometimes aqueous media must be used, and sometimes oils or resinous substances. A great deal can be done to decrease the effect of obstructive portions of a specimen by careful selection of the mounting medium. For direct recommendations consult the sections describing the characters of the different media and the special section appropriate to the plant under consideration.

The following table has been adapted from Schneider-Zimmerman.<sup>28</sup> Those media marked with an asterisk require dehydration.

Substance	Refractive Index Approx.
Air .....	1.000
Water, distilled .....	1.333
Alcohol, ethyl. ....	1.361
Formaldehyde, 40 per cent. ....	1.372
Glycerin, 50 per cent aqueous .....	1.400
Lactic acid .....	1.441
Glycerin gelatin .....	1.447
* Linseed oil .....	1.470
Glycerin, pure .....	1.475
* Paraffin oil .....	1.481
* Xylol, toluol. ....	1.495
* Cedarwood oil, clearing .....	1.504
* Cedarwood oil, immersion .....	1.515
* Clove oil .....	1.535
* Euparal .....	1.535
* Wintergreen oil .....	1.536
* Venetian turpentine, conc. ....	1.542
* Canada balsam, hardened .....	1.547
Zinc iodide, sat. glycerin sol .....	1.560
* Styrax, hardened .....	1.580
* Balsam of Tolu, hardened .....	1.640
* Monobromnaphthalein .....	1.661
* Hyrax .....	1.822

<sup>28</sup> Schneider-Zimmerman. Botanische Mikrotechnik, 1922.

2. **Aqueous mounting media** as used for plant epidermis, hairs, fibers, pollen, spores, unicellular or filamentous algae or other small objects generally give slides of a fair degree of permanence if the cover glass is suitably sealed to the slide (p. 199).

Parts of moderate thickness had best be placed with the fluid in a ring-like cell built up of several well-dried coats of cement and the cover sealed to this. The fluid used may be a 4 per cent aqueous solution of formalin, 2 per cent aqueous acetic acid or 10 per cent aqueous glycerin (or a stronger glycerin if the object will stand it). Glycerin of 10 per cent strength is not immune to the growth of molds; if a crystal of thymol is kept in the dispensing bottle these organisms will not develop. Marine algae if mounted from the living state should be put into a solution compounded with sea water, rather than distilled water. Lime impregnated material (as Characeae) must not be sealed in acetic acid, and is safest in glycerin.

It is possible to preserve tolerably well the color of many green algae, including Conjugales, Ulotrichales, etc. The simplest fluid consists of concentrated formalin which is saturated with copper acetate. If this is diluted to about 4 per cent it makes a very satisfactory preserving fluid for blue green or green algae in which they may be kept in bulk or in which they may be mounted on slips. The green color will shift to a somewhat more bluish cast than the original, and some shrinkage may occur.

Much botanical material of a type demanding mounting in aqueous media for its most perfect exhibition should be mounted in a medium of a higher refractive index than those just mentioned. In general the material should be brought into these more dense media by suitably graded changes. The most generally used fluid will be concentrated glycerin. The material may be placed in a 5 per cent to 10 per cent solution and this concentrated by evaporation in a warm place. A solution (Lactophenol) composed of lactic acid 20 gm., carbolic acid (crystals) 20 gm., glycerin 40 gm., water 20 gm., is very serviceable and may be used for mounting various materials, softening dried material (especially algae) or decalcifying specimens. A dye may be combined with this solution. Maneval<sup>29</sup> has suggested several combinations, of which that involving the addition of 1 to 5 c.c. of 1 per cent aqueous cotton blue and to 20 per cent of acetic acid seems to have the widest utility. Zinc iodide saturated in glycerin gives a solution of very high refractive index, and has been used for diatoms.

Stains suited for use on material to be mounted in fluid are not very numerous. Preserved material may be permanently stained in 1 per cent

<sup>29</sup> Maneval, W. E. *Stain Technology*, 11: 9, 1936.

aqueous eosin solution, differentiated in distilled water, the stain then fixed with 2 per cent aqueous acetic acid, in a fresh portion of which the material may be mounted. The nuclei and chromatophores will be bright pink, other cell contents paler. It is possible to transfer material to very mildly acidified 10 per cent glycerin and concentrate this, but the stain usually fades. Preserved material may be carefully stained in Delafield's hematoxylin, well washed in tap water and mounted in dilute or concentrated glycerin. Cellulose membranes will be light purple, nuclei and chromatophores darker; the stain is fairly permanent. Preserved material may be mordanted in  $\frac{1}{2}$  per cent aqueous iron-ammonia-alum, washed, stained in  $\frac{1}{2}$  per cent aqueous hematoxylin, rinsed and exceedingly cautiously destained in  $\frac{1}{2}$  per cent (or even more dilute) iron-ammonia-alum, washed and mounted in dilute or concentrated glycerin (see below). The stain is permanent; nucleoli and pyrenoids stain black, nuclei and chromatophores gray. It is highly satisfactory and on many forms (such as *Spirogyra nitida*) strikingly effective.

It has been found that corn syrups if stable yield good mounting media for Bryophyta. They dry gradually near the edge of the mount and do not need immediate ringing with cement. The "Blue Label Karo" is particularly satisfactory for this purpose. Water glass (sodium silicate) may also be used but is not as reliable. A proprietary mixture in glycerin called "Viscol"<sup>30</sup> will serve the same purpose very well and may be used after water, glycerin or lactophenol.

Since in fluid material it is difficult to maintain the orientation of unicellular organisms and since many fluids dry out if carelessly sealed, a series of solid media have been designed. The most commonly used is termed glycerin jelly and is applied in a melted state. When a large amount of material is to be mounted the bottle may be kept in a hot water bath, but for occasional use it is better to melt small blocks of the jelly on each slide and add the material to the melted medium, thus avoiding a continuous decrease in the gelatification power of the gelatin. A satisfactory formula consists of high grade dry gelatin 15 gm., distilled water 60 c.c., glycerin 70 c.c., carbolic acid about 1 c.c. The carbolic acid and the glycerin should be mixed and added to the gelatin dissolved with heat in the water. No ordinary stains can be depended upon as permanent in this medium. Material which shrinks easily should be transferred from strong glycerin.

*Cements for sealing aqueous microscopic mounts* of plant tissues, fibers, etc., in watery solutions, glycerin or glycerin jelly are important factors in assuring permanence of these preparations. The cover glass and the slip about it must be scrupulously clean, the faintest trace of

<sup>30</sup> Amann, J. *Rev. Bryol. et Lichénol.*, 8: 188, 1935.



glycerin causing the slide to deteriorate rapidly. Thin coats of the cement are brushed over the margin of the cover and the adjacent slip free-hand or with the aid of a turn-table, each being allowed to dry before its successor is applied. Various substances and mixtures have been suggested, including vaseline, shellac, marine glue, King's cement, gold size, asphaltum, etc. A particularly satisfactory medium suggested by Hazen is composed of rosin 8 parts and anhydrous lanolin 2 parts, the rosin being melted first and the lanolin then added. This may be applied hot to the edges of the cover glass with a glass or metal paddle, or dissolved in benzol or toluol and applied with a brush. It does not crack or become brittle under ordinary conditions. If it is to be used to seal slides kept at a low temperature the amount of lanolin may be raised, or if used in the tropics it may be decreased. It may also be used to seal vials or museum jars with a slight increase in lanolin content.

3. **A non-drying oil** is sometimes a desirable mounting medium. For this purpose white mineral oil or paraffin oil is most suitable. It requires dehydration and clearing of the material through xylol, benzol, etc. Mounts in this may be sealed for a time with glycerin jelly or glue. Oil of wintergreen is rather better in that clearing is not necessary, but it has a higher refractive index. The synthetic form, methyl salicylate, is quite satisfactory and may also be used for clearing injected stems, leaves, etc., for gross anatomical observations. Castor oil, linseed oil, olive oil may also be used for temporary mounts. Glycerin jelly may be used to seal such mounts.

4. **Resinous mounting media** serve in general the same purposes for plant and animal tissues.

Damar balsam, or gum damar, is a very excellent and economical mounting medium, and one which should displace Canada balsam. The crude product as packed by dealers generally consists of vari-sized lumps and powder. Most of the trash is mixed with the finer material, so that if the lumps are picked or screened out a notable saving is accomplished in subsequent clarification. A general method of preparation, then, is to discard powder and trash, rinse off the lumps with xylol, pick out any dirty ones and dissolve the clean remainder in xylol, or if a more rapidly drying reagent is desired, in benzol and filter. Some difficulty may be found in filtering off the trash, and the product may not be perfectly clear. This is not serious in itself, but as users of this mixture used to report a little crystallization of the medium in old slides, it may be symptomatic of other defects. The writer offers an alternative method which he hopes has eliminated both difficulties, for preparations made ten years ago are consistently found to be in perfect condition.

The crude material, preferably only the lumps, is melted over a

Bunsen flame. The fluid product is poured into an ample volume of the ultimate solvent (benzol preferred) with constant stirring, to produce a quite weak solution. When the coarser trash has settled the balance is decanted through a loose plug of glass wool in a funnel, then filtered through a couple of thicknesses of lens paper in a ridged funnel, and finally through a soft filter paper. The main difficulty in filtering seems to be due to a slimy flocculent contamination which settles with extreme slowness. However, if dilute, the solution passes very quickly through the strainers indicated and yields a perfectly clear, pale amber fluid which, since it is too dilute for most uses, should be allowed to concentrate in a warm place. If a little more of the flocculent precipitate separates out it can be discarded by decanting. A large batch of the medium can readily be prepared in an afternoon by this method. This solution is freed by the preliminary heating of any moisture which may have been contained in the resin, and the final solution is much cleaner than by the alternative method. Damar balsam offers the important advantage of resisting marginal oxidation around the preparation so that hematoxylin slides do not fade. Aniline dyes are perfectly safe also, and as damar is much less yellow than Canada balsam, blue dyes show to better advantage in thick mounts, and for photographic purposes it offers less of a color-filter action to disturb actinic adjustments. (For algae, etc., see p. 226.)

When mounting delicate filamentous organisms (as algae) the damar should be diluted with 10 to 20 parts of benzol or xylol and the specimens placed in this diluted mixture to concentrate by evaporation. This method will avoid the shrinkage resulting from placing a resinous mounting medium in full strength directly upon such organisms.

Canada balsam, as sold for student use in collapsible tubes, is often, even generally, simply the natural resin filtered free from debris. It contains turpentine and other substances harmful to stains and what is even more troublesome, oils that evaporate slowly, so that the mounts made with this product (usually called "paper filtered Canada balsam") harden only after many months. For proper use this raw balsam should be gently heated over a flame or sand bath to a temperature that will volatilize these hydrocarbons so that the mass, when cool, is quite hard. It is then dissolved in xylol, toluol or benzol, the last evaporating rather more readily than the xylol. Chloroform is to be avoided because it fades some aniline dyes. The solution used for most plant histology, especially where the sections are rather thick, should be like thick cream. For celloidin and paraffin sections, as for most zoological work, the solution should be much thinner. It should harden firmly around the edge in twelve to twenty-four hours (less for thin mounts). The universality

of adoption is one of the chief recommendations of this medium. When critically inquired into it is found open to serious objections. If the heating has been insufficient volatile oils remain that are dangerous to aniline dyes. Mounts at first satisfactory gradually deteriorate centripetally as yellowing and oxidation of the medium progresses inward, for this acid condition readily fades hematoxylin preparations and some aniline dyes. A peripheral third or more of a cytological mount may thus be ruined in five to ten years, and where such slides have value as records this is very serious. For filamentous plants a special technique is most effective (p. 229).

For mounts which it is desired to retain in a rather soft condition for a considerable length of time raw Canada balsam may be used, or thickened cedar oil, or Venetian turpentine (p. 203). The latter does not require clearing before mounting nor does the mixture sold under the name of Euparal, which is a very useful mounting medium entered from alcohol of 70 per cent (?) or higher. Euparal does not seem to fade dyes, but is liable to cause considerable shrinkage in any plant material whose cells are not laid open by a sectioning device (as *Fucus* sporelings, algal filaments, etc.). For work with diatoms where it is desired to secure the maximum refractive index possible, styrax or tolu balsams are often used. They may be prepared as damar by the cold method, and are usually dissolved in chloroform, which allows the slide to harden quickly. Recent investigations by Hanna<sup>31</sup> show that he has succeeded in preparing a permanent resinous medium synthetically which has a refractive index of 1.70, exceeding 1.82 when hardened. It is soluble in xylol or benzol, and has a somewhat yellow color, but is excellent for work on diatoms or chitinous structures.

*Balsam mounts are cleaned* after they have hardened, when it is customary to clean around the cover glass by scraping and wiping with xylol. A better method is to prepare a mixture of equal parts of xylol and 95 per cent alcohol, and then add water to this clear mixture drop by drop with shaking, stopping just short of milkiness. If too much water is added the alcohol and xylol will separate, which requires that the bottle be shaken frequently during use. If this separation or milkiness occurs add a few drops of absolute alcohol and shake until the mixture again just becomes clear. Keep in wide-mouthed bottles and dip slides in the solution until the excess balsam is softened enough to wipe off. If the mounts are not unusually thick this fluid will not show a tendency to cut under the cover in normal use.

**5. Balsam infiltration mounting method** is an alternative to the Venetian turpentine method (below) and much preferred to it by the

<sup>31</sup> Hanna, G. D. *Science*, 65: 575, 1927. *J. Roy. Micros. Soc.*, 50: 424, 1930.

writer for filamentous algae and fungi, prothallia, etc., and is a method long since adopted by various Continental workers handling this type of material. The filaments are stained in Heidenhain's hematoxylin, safranin and light green, safranin and aniline blue, phloxine B and aniline blue or other suitable combinations (p. 185). From aqueous stains they are placed in 5 per cent glycerin and this concentrated by evaporation. From stains in alcohol, dehydration is usually completed through alcohol. The concentrated glycerin is removed very carefully (if necessary) by several changes of 95 per cent alcohol. It usually sticks to the mass rather closely. Two or 3 changes of absolute alcohol follow, then a series of about 6 to 10 intermediates between alcohol and xylol (as: 5:1, 4:2, etc., or 9:1, 8:2, etc., the longer series being preferred). The time in absolute alcohol and these xylol mixtures can usually be reduced to five minutes or less; they are followed by 2 or 3 changes of pure xylol. It is then placed in dilute Canada, or better, damar balsam, in strength about  $\frac{1}{4}$  or less of that used for ordinary mounts. This is allowed to concentrate by evaporation at room temperature or near a radiator. The transfer to the balsam is somewhat critical; this must be very dilute. The writer found greatest liability to shrinkage at this point, but the remedy is simple. Evaporation to a mountable consistency should take about two days (rarely more) for delicate material (*Spirogyra*, *Vaucheria*). The filaments should be dipped up with a section lifter and placed on the slips with the solution in which they have been prepared. If the volume is insufficient it may be augmented with a little more dilute (on no account more concentrated) balsam. The mounts harden quickly and are altogether permanent. Advantages over the Venetian turpentine method lie in the avoidance of the very critical alcohol-turpentine transfer with the danger from water vapor and the bulky desiccators; in the more rapid hardening of the mounts; in the wider choice of stains available; in the more ready availability of the medium and its somewhat higher refractive index, etc. This method is extremely good for all delicate material. Vegetative *Spirogyra*, for instance, should show no shrinkage, while even late conjugating stages come through exceptionally well, although it is very difficult material.

6. Venetian turpentine infiltration mounting method has been so capably presented and praised by Chamberlain<sup>32</sup> that special commendation is superfluous. The writer considers the balsam infiltration method to be distinctly preferable. The process follows that method to the absolute alcohol stage. The turpentine is used as a 10 per cent solution in absolute alcohol. Material is placed in it from absolute alcohol in an open dish and immediately put into a desiccator over soda lime. Here it be-

<sup>32</sup> Chamberlain, C. J. *Methods in Plant Histology*, 1924, p. 101.

comes concentrated to a mountable consistency. The turpentine at 10 per cent is excessively sensitive, absorbing atmospheric moisture and clouding readily. The denser solution in which the material is mounted is not so sensitive. This method eliminates the xylol-alcohol stages, but has various disadvantages (p. 202). Exceedingly successful results may be achieved by it.

## B. CYTOLOGICAL METHODS

### VI. Choice of Methods and Standards

Choice of methods and standards by which the results may be judged constitutes the most difficult problem in cytological technique. It is no longer possible to give a concrete schedule which by minor variations can be considered as covering the requirements of studies in plant cytology. It is not enough to consider what plant is to be the subject in planning a technique, nor what organ of it, nor the particular cells and their structural positions, but one looks to the cell organs of special interest and their physiological state, usually as it may be marked by a mitotic phase or some other condition associated with a distinctive morphological picture. Since, with the newer developments in using chromosome numbers as distinguishing features in genetical work, a vast number of published data have been based on studies using technique of the most primitive character, it becomes necessary to distinguish much more sharply than has been done in the past between processes which promise to produce results of fundamental importance in pure cytology and those which only subserve chromosome counting and similar utilitarian purposes. Studies of the latter class will assist geneticists, but they will not advance the basic knowledge of cell processes. Although the difficulties of making such studies may be considerable, and although there may be physical obstructions in the way of easy observation, yet they will always rank as requiring a lower grade of effort and method, less knowledge of general cytology and less constructive imagination than studies from which knowledge of a process rather than a mere list of figures is the ultimate result.

It is not possible, naturally, to mark a sharp line of distinction between critical and applied cytology, but it is vitally important to indicate the wide range of results required and of methods appropriate to these. Material which has been prepared by methods suited to the mass production of modern utilitarian data, counts, etc., that hardly more than serve to relate the plant as represented by one cell organ to other plants, is rarely, if ever, suitable as a source of information on basic conditions

and changes in the cell as an organism or an organ. This cannot be too strongly emphasized. It appears to the writer most unfortunate that persons familiar with the methods suitable for counting purposes should have so freely fallen into the erroneous idea that because the cells appear unshrunk and the chromosomes distinct at metaphase then all other nuclear division stages (not to mention cytoplasmic features) must be correctly portrayed. As a matter of fact this is far from true; in hardly any of the recent studies in which chromosome enumeration was the prime desideratum are the other cell features when described (chromosome form, prophase and anaphase structure and history, all cytoplasmic organs) even approximately truthfully interpreted. It is quite impossible for a man in two or three years to count the chromosomes in scores of individuals (not to say species), to do so by methods which would correctly preserve the more critical phases and still have time to observe intelligently these conditions, interpret them in scholarly fashion and record them in a clear style. The attitude of mind which would lead him to undertake the counting of a long range of forms would prohibit the more critical labor, even though a technique may be perfected (p. 166, new smear method) which will enable him to accumulate quickly a great mass of critical material. In no case is it safe to draw even the more elementary conclusions as to the cell history from material fixed after the old style methods, involving unopened or even opened flower buds, large unopened anthers, cells in the depths of root tips or comparable conditions in other than phanerogam plants.

A primary distinction must be drawn between three types of cytological study, viz., those of cytosomic, mitotic and nuclear elements. First come those involving the general structure and the vegetative organs of the cytoplasm. These methods involving the structures (or appearances) in plant cells called Golgi apparatus, mitochondria chondriosomes, etc., are studied by methods not essentially different from those used on animal cells, for which most of them were designed. Consequently reference to these methods should be made (p. 265).

1. **Flagella** are generally most readily demonstrated by methods adapted equally to the motile stages of algae or to protozoa. Dark field illumination is highly effective. Fixation of organisms in a small hanging drop by osmic acid vapor followed by drying of the sample and staining by Loeffler's bacteriological technique (p. 142) is often effective. Simple fixing and staining under a cover glass with aqueous iodine is also often effective.

2. **Protoplasmic connections (Plasmodesmen)** between cells are generally made more evident by the swelling of the walls. They are most suitably demonstrated in algae (p. 235) or in endosperm of seeds, sieve

tubes, cortical tissues of some gymnosperms, etc. They may be fixed adequately with simple 1 per cent osmic acid and stained with crystal violet or pyocyanin (3 per cent aqueous). If the walls are to be swollen this may be effected with 25 per cent (or stronger) sulphuric acid to which iodine may be added.

3. **Chromatophores** (including chloroplasts and leucoplasts which have essentially the same structure) are legitimately discussed here. In studies attempting to trace their origin from mitochondria-like bodies methods from page 265 should be selected. Comparatively little information as to chromatophore structure appears to derive from preparations using the standard Flemming's fluids, Bouin's and Gilson's mixtures, etc. but they are quite satisfactory for studies on chromatophore form, especially the first group, provided the fluid acts quickly enough to prevent contraction of the plastid.

4. **Pyrenoids** show quite satisfactorily after these same fluids and their position in relation to starch grains, etc., may be determined. Material fixed in saturated alcoholic bichloride of mercury and thoroughly washed may be stained for twenty-four hours in 0.2 per cent acid fuchsin, when the pyrenoids should be bright pink, the nucleoli by contrast not stained. As the chromatophores are quite responsive to toxic agents it is essential that killing be very rapid to prevent these contraction alterations. After fixing, washing and dehydration follows a moderately close schedule (p. 161). Some pertinent data have recently been presented by Zirkle<sup>33</sup> who has worked with monochromatic light on living chromatophores.

5. **Eleioplasts** may be fixed like chromatophores, but special methods have been suggested. Material fixed in saturated aqueous picric acid is stained in a solution of aniline blue in water which has been turned from dark blue to a Delafield's hematoxylin-like purple by the cautious addition of alkanin. After several hours the eleioplasts should show purple, contrasting with the light blue of the plasma, the dark blue of the nuclear chromatin and the red of oil droplets. In neutral glycerin gelatin the colors hold fairly well. As the exact method of functioning of these structures is obscure there is need of more intensive study of them, and the student should refer to the literature for other methods.

6. **Polar caps, spindle fibers, cell plates, asters, centrosomes and blepharoplasts**, constituting a second type, come in a group of cytological structures that appear during cell division. The last two are often peculiarly responsive to osmic acid, failing to be visible, for instance, in chrom-acetic preparations, but with the osmic acid coagulating firmly. A good example is the spermatogenous cells of *Ginkgo*, where the big

<sup>33</sup> Zirkle, C. *Amer. J. Bot.*, vol. 13, 1926.

spherical bodies that organize the blepharoplasts stain deep black after chrom-osmo-acetic but appear only as empty cavities after chrom-acetic. On the other hand polar caps and spindle fibers are of reduced visibility in preparations where osmic acid is present in large amount. The fibers are probably excellently preserved. The trouble lies in the fact that in these the cytolymph is much more completely precipitated than in the absence of osmic acid and so the fibers no longer stand out against a contrasting background. If studies are being made on the organization of the spindle by a chrom-osmo-acetic fluid then one should reduce the osmic acid as far as possible, following fixation and washing by dehydration and clearing with a moderately close schedule (p. 162). If the reduction of osmic acid is carried to excess the cytoplasm will appear as a mass of coarse vacuoles due to the disappearance of the most bulky portion of the cytoplasm. The irregular coagulation of the remainder will distort the fibers and give a false impression of the positions of these and of the other cell contents. Especially during mitosis and meiosis is this important, because lacking the support of the more completely coagulated cytolymph the chromosomes become clumped together. This appears to be the reason why persons working with plain chrom-acetic solution or other fluids that incompletely fix the cytoplasm (which are often used in chromosome-count studies because of the ease in getting a brilliant stain) report that the chromosomes are closely clumped together even when the cell offers ample room for the metaphase plate to spread out.

**7. Nuclear and chromosomal structures**, the subjects of the third type of study, can in certain features be preserved well even though the cytoplasm is badly fixed. On the other hand many elements of cytoplasmic structure cannot yet be preserved by any methods suitable for nuclear organs, although conserved readily enough by their own special technique. The feature of cell construction which is most notably upset by imperfect cytoplasmic coagulation is the space relation between the formed elements. For instance, unless the cytoplasm is well and finely precipitated the chromosomes tend to clump together, as was mentioned above. This is a rather obvious phenomenon the danger of which is far too often discounted. The little rounded chromosomes which are usually reported as practically touching probably do not normally approach each other closer than to one-half their own diameter. Completeness of fixation of the cytoplasm avoids this artifact and enables counts to be made much more readily.

Organic adjuvants, especially sugars of which maltose and saponin appear the most adaptable, seem to assist in preventing clumping (p. 218). They also assist in maintaining the space between longitudinally cleft chromosome and spireme halves, and have contributed toward a



clear recognition of the chromosome tetrad in plant cells. This may come from expediting the penetration of the fixing fluid. With the clumping of the chromosomes is associated a tendency for their contraction, most visible of course, longitudinally. While obviously more rigid than the surrounding materials, they are distinctly responsive to changes in the fixing fluids, and readily contract if slowness of penetration or incompleteness of coagulation enable them to do so. There is no proof that this effect is necessarily equal on all chromosomes in a given complex, so all studies involving a comparison of chromosome lengths must be conducted with the utmost precaution to prevent this contraction. It is not practicable to judge this directly, for the absolute lengths of chromosomes of a given kind vary from cell to cell even within a single kind of tissue depending on the stage and rapidity of mitosis or other factors, and even more between different kinds of tissues. The easiest guide to a recognition of such a change is the critical observation of the state of the fiber attachment or other constrictions, and of the satellites. If these are quite distinct, and the fibers attaching the satellites are not shortened, there is good reason to feel that the chromosomes themselves are in normal condition. Even for this some standard of excellence is necessary, but a matured experience guided by these criteria is less likely to err than in judging contraction directly, where the normal for a given kind of cell may vary greatly.

To what degree constrictions of various kinds are a normal chromosome character is not wholly clear, but it appears that they are present at least in any chromosome large and long enough to exhibit them at the region of fiber attachment and in frequent cases may appear elsewhere. They certainly are the morphological expression of one phase of a longitudinal differentiation of the chromosome, and experiments with chloral hydrate have demonstrated a rather extensive, if ordinarily obscure, continuance of this differentiation.

Furthermore, direct observation of living cells may be employed (in favorable cases) to gain an idea of the natural conditions. It is worth while, however, to direct a warning remark against undiluted faith in observations on unstained (including living) material, or material under weak intra-vitam stain. The technique of such observations is discussed elsewhere (pp. 110, 117, 281). The difficulties of interpretation are great, and require a special training not furnished by experience on stained material in resinous media.

The remarks that have been emphasized on the importance of a complete fixation of the cytoplasm for general studies and particularly for studies on the chromosomes during cell division apply well regarding the nucleus in maintaining the relation between its parts. The following

statements, although specially adapted to such laboratory types as *Allium*, *Galtonia* or *Fritillaria* and to root tip cells, are really quite generally applicable. It is perfectly obvious on an inspection of the drawings accompanying a great many recent cytological studies that the fixation of nuclear material was limited to the nucleolus and the spireme or its representative granules. The rest of the nucleus in the fixed preparation is empty. It is far from this in the living state, being filled with a quite coagulable karyolymph. To expect the chromatic elements of the nucleus to maintain their proper relative positions without the support of this matrix would be obviously absurd. It must therefore receive careful consideration in judging the value of the fixing fluid used. If the precipitation is too dense it will be hard to secure a satisfactorily differential stain of the finer chromatic elements. Therefore the karyolymph should be coagulated in a fine-grained soft mass rather than the heavy refractive mass which comes, for instance, from an excess of osmic acid. If this is properly effected very little displacement of the contents of the nucleus will occur. It will be found that the apparent peripheral state of the granules of the resting nucleus and of the early spireme stages is only an artifact and that they are generally disposed through the nucleus.

The nucleolus will not show a bubbly structure, but will usually exhibit a smooth texture with sometimes one or more darker bodies. It will not be surrounded by a clear space, which is simply due to the shrinkage of the other nuclear contents from it, generally because of inadequate fixation of the karyolymph. This artifact is in itself conclusive proof of bad preservation of the nuclear contents. Inspecting the spireme one is able to recognize its duplex character far forward into the earliest stages, in fact probably as far as its thread-character can be traced. In somewhat later stages segmentation of the spireme and the first traces of the constrictions can be seen. In the cytoplasm the polar caps will be recognizable and the clumping of the chromosomes usually associated with the breaking down of the nuclear membrane will not be very great.

The amount of differentiation which it will be possible to demonstrate within the metaphase or anaphase chromosomes is problematical and more within the province of a research paper than a general introductory statement such as this. With discretion respecting the fixing fluid and the infiltration, it should be possible (especially with a curtailed hematoxylin staining schedule) to recognize a good deal of specialization. It appears probable that at least in the larger chromosomes in vegetative anaphase there are spiralled threads sometimes bearing chromomere granules.

A few further general suggestions may be offered respecting cytological criteria in meiotic and post-meiotic material. The hematoxylin smear

method (p. 166) has confirmed several features of chromatin distribution previously in more serious question, so that the general standard to be demanded of preparations of such stages is higher than a few years ago. In the resting stage and very early prophase the chromatin material usually does show a general distribution throughout the nucleus, even in the absence of a good preservation of the karyolymph. Since that substance is more readily coagulable at this time than later, many kinds of cells show quite respectable preservation in a fluid that is not adequate for later stages. However, very careful attention to this phase and the use of a fluid more effective with the karyolymph notably reduces the confusion and indefiniteness of distribution of the very early spireme and apparently granular stages and offers good prospect of an eventual complete interpretation of them. It is to be expected that a spireme will be recognizable far into the prophase toward the period of the first concentration of the chromatin. This spireme (at least in large spore or pollen mother-cells) will be seen to be double. It is to be hoped that in later stages (strepsinema, etc.) more cases of a tetrad structure will be demonstrated than are at present known. It is possible to expect a great reduction or even (in cells not too resistant to fixing fluids) an elimination of the contraction feature known as synizesis (by botanists often "synapsis") which represents simply the collapse of the spireme by reason of lack of support of the coagulated karyolymph at a time when the spireme is particularly slender and delicate. The conditions proper for a truthful exhibition of spireme and pre-spireme conditions (if the latter ever exist) are notably absent from most preparations of meiotic cells, for the correct preservation of them is far from the casual matter generally assumed. It must be understood that pollen or spore mother-cells discussed in the preceding paragraphs have been preserved without shrinkage of the cytoplasm, or practically so. It is almost impossible to conceive of accurate nuclear studies being made on material where even the cytoplasm surrounding the nucleus, easier by far to conserve properly, has not been able to resist malformation by a badly designed fixing fluid. If the pollen mother-cells cannot be exposed to the fixing fluid directly they will generally shrink, and in the case of small anthers or sporangia little can be done about it. As such material is unsuitable, if the problem contemplated involves detailed and critical observations other species of plants should be selected.

In the case of embryosacs and deeply immersed megaspore mother-cells of conifers a comparable situation holds. It is practically impossible to get a fixation of these suitable for really critical work. What suggestions can be made are offered on page 214. The stages secured will have to be presented for what they are worth, without offering them as evi-

dence on matters of critical importance respecting spireme constitution and behavior. It has frequently been considered by plant cytologists that they have observed transverse division ("fragmentation") of rather elongated chromosomes, and various interpretations have been made of this process. Where there is opportunity to judge it seems that the lines of division are sharp, the ends square-cut, and not constricted across as one would expect. It may confidently be assumed that these always represent fractures due to imperfection of the sectioning process: too brittle paraffin, dull or ragged-edged knife, etc. It is hardly likely that they are anything but artifacts. The positions in which they appear are fortuitous rather than definite like the chromosome constrictions. Chromatin extrusion from the resting nucleus or the early spireme stages is also an artifact due perhaps to pressure on the tissue in cutting or some other early stage of preparation.

## VII. Examination of Living Cells

The study of living cells, usually pollen mother-cells, in search of cytological data has resulted in contradictory reports, particularly with respect to the details of mitosis. Somewhat more satisfaction has resulted from the use of intra-vitam dyes, but here the actual health of the cell has been much in doubt at times. (For a discussion of the methods applicable to plant problems see p. 212.) The morphology of the colored elements of the cell, chromatophores, eyespots and the like, may best be determined in the living state, and require no special methods. Chromatophores with dilute pigmentation may be made such more evident and studied much more readily if a suitable ray-screen is placed below the condenser. A ready selection may be made from the complete and accurate series prepared by Wratten & Wainwright and distributed in the United States through the Eastman Kodak Company. It appears that an important new approach to the effective observation of living cells may result from the studies of Kuwada and Sakamura<sup>34</sup> and Sakamura.<sup>35</sup> These investigators found that the acidity of the medium in which the observations are made has a very striking effect on the distinctness of the chromosomes and of their structural elements. They found that there is a general tendency for the chromosomes to swell as the pH of the medium passed from 1.7 toward 6.7, and that this is, within wide limits, a reversible reaction. As the chromosomes contract they and their structures become more visible (in the living state) and as they swell these disappear, so that the chromosomes at metaphase appear to be represented by

<sup>34</sup> Kuwada, Y., and Sakamura, T. *Protoplasma*, 1: 239, 1926.

<sup>35</sup> Sakamura, T. *Ibid.*, 1: 537, 1926.

hollow cavities in the cell showing little content, even with dark-field illumination. Observations may be made in the viscous material from the anther cavity if that is abundant enough, or the anther squeezed into a drop of olive oil and this pressed out under a cover. In this latter method few of the pollen mother-cells actually come into contact with the oil, the rest remaining surrounded by the material from the anther cavity, even when it is small in amount. If  $\text{CO}_2$  is passed over material under observation it is found that as the gas is absorbed by the cells the obliquely transverse banding of the chromosomes becomes increasingly evident, and as the gas is driven out of the drop by a current of  $\text{CO}_2$ -free air the chromosomes swell and the structure becomes indistinguishable. For *Tradescantia* it was found that a pH of 5.0 (or a little more acid) was about correct for a well buffered medium comparatively insensitive to the action of alkali. The anther content is distinctly alkaline and readily affects a lightly buffered suspension medium. The experimenters got the best results in demonstrating the spiral structure of the chromosomes of *Tradescantia* when the dye, neutral violet extra, was dissolved to .025 per cent in a solution of pH 4.38, and observations made on the peripheral units of the masses of pollen mother-cells. They consider that the spiral structure of the chromosomes is a natural feature easily lost from view by the swelling of the chromosomes or accentuated by their slight contraction. The spirals may be fixed in various ways, even by simple boiling water.

*Intra-vitam stains* have met with limited use on plant material due to the firm nature of the cell wall, the frequent presence of pigments, cutin and so forth. However, when the nature of the organism permits, a procedure very similar to that available for animal cells can be used. Root hairs, larger aquatic plants and aquatic algae and fungi can simply be immersed in aqueous solutions of the dye chosen. Hairs of pistils, stamens, leaves, etc., and stripped epidermal layers can usually be kept in good condition and stained in an isotonic solution. Sometimes suitable staining of vascular plants can be secured by immersing the cut ends in a fairly strong solution. Perhaps this is hardly strictly "intra-vitam" staining in the cytological sense, although cells bordering the vessels do take up some dyes. If eosin is used and the plant is sufficiently tender (e.g., *Impatiens Sultani*) the entire stained shoot and leaves may be plunged in 2 per cent acetic acid in absolute alcohol to dehydrate and fix the stain and then after a few hours transferred to oil of winter-green (synthetic) to clear the preparation. For the accurate staining of living cells only a limited choice of dyes is available, of which the most important are methylene blue or neutral red (1:10,000 to 1:100,000) and bismarck brown (1:3,000). They should stain nuclei, chromatophores,

etc., and it will often be found that the stain becomes more intense as the cell becomes injured by the treatment (p. 211).

### VIII. Preparation of Cytological Material for Fixation

This requires close attention to all possible ways of facilitating the action of the fixing fluid. Under general paragraphs appropriate to each major group of plants there have been assembled suggestions as to technological methods specially suited to them. Certain general precautions should always be borne in mind and will be discussed here. Although problems in connection with vascular plants have been used as a basis for wording these directions, the data may be adapted to other groups with little modification.

1. **Root and stem tips** should be freed from sand and loam, and if they are covered with a mucilaginous investment it should be washed away if possible. The tips should not be more than 1 to 2 mm. in diameter at the very most, and if thicker, slices should be cut off opposite sides with a very keen piece of razor blade to reduce the axial portion to about 2 mm. so that the time for complete penetration should not be unduly prolonged. The mucous layer of air roots, or in other kinds the suberized root caps, can be eliminated in this way. The stem tip should be trimmed free from the leaf bases, rammenta or hairs. The desired portion should then be cut from the plant, detaching as small a part as practicable; certainly not more than 3 to 5 mm. long. In root tips it will generally be found that there is a meristematic region just back of the apex that is visibly more milky or yellow-opaque than the cap beyond it or the region of elongation above it, and this region contains all the material that is worth while for ordinary cytological purposes. The tips should each be plunged below the level of the fixing fluid instantly after cutting; indeed if any trimming is necessary it is well to do it under the level of the fixing fluid.

2. **The study of the meiotic phases** by fixing buds, anthers or ovules intact has long been the favorite method. With the increasing requirements of modern cytology for freedom from shrinkage, from synizesis, from chromosome clumping and contraction and for preservation of the internal structure of the chromosomes, such crude methods must rapidly fall into disrepute. In very tiny flowers it may be impossible to open the buds and these will then have to be fixed entire. Hairy buds may be dipped into 50 per cent alcohol for an instant to remove the air, and this sometimes helps when there are cuticularized surfaces which cannot be cut away or otherwise removed, but after alcohol they must be quickly plunged into the fixing fluid. This treatment should be omitted before

using Farmer's or Carnoy's fluids, or other formulas with a considerable alcohol content. Buds and capsules of lower plants containing air chambers should generally be placed, immersed in the fixing fluid, under a vacuum pump to remove all possible air. If it is possible to do so the flowers should be bisected, or a piece cut off one side. A mere longitudinal incision or a needle puncture is hardly effective. If the anthers are 1 mm. or more long they should be carefully excised or the perianth trimmed from about them. At 2 mm. or more they can be cut across transversely, and if they are several millimeters long they can be cut up into small fragments, or handled by the smear methods (p. 165). For holding the fixing fluid while cutting, the writer uses a Syracuse watch glass with a layer of paraffin on the bottom. About 5 c.c. of fixing fluid is poured on the wax and into it are dropped the anthers, each cluster being immediately finely comminuted with a small, very sharp, scalpel of the size used for eye operations. The accumulated anther fragments and all extruded materials are then handled through the paraffin process by a sedimentation method like unicellular organisms, which is really very easily done. Truly superb fixation of meiotic cells may be obtained in this way, the quality approximating that afforded by the smear method (p. 166) with the advantages of ease in staining and of serial sections. The secret of success lies in bringing the cells to be studied into immediate contact with the fixing fluid. As the cells are squeezed from the anther they adhere to the cut surfaces, the walls in the sacs, etc., or even to each other so as to form considerable masses. In fact, in some plants the sac contents squeeze out in a rod-like mass when pressed from the anther, and if it is considered desirable they can be fixed, dehydrated, imbedded and cut in this form, yielding admirable results, as has been demonstrated to the writer by E. E. Carothers for *Lilium*. The exposed grains yield infinitely more accurate pictures of true conditions than do cells buried even one or two layers from the surface. In the larger fragments it is easy to trace comparatively the difference in fixation quality from these exposed cells down the anther sac to the deeper-placed cells that show (in quite the most favorable way) the orthodox fixation to which plant cytologists have unfortunately become habituated. It follows from these remarks that studies on meristematic tissues, such as root tips, should be confined to cells very near the surfaces of the pieces, not more than 2 or 3 cell layers removed from direct contact with the fixing fluid, if observations of great nicety are to be made.

*Ovaries* offer a worse situation. The problem is the same: to give direct contact between the fixing fluid and the embryo sac. In such ovaries as those of *Liliaceae* the walls should be dissected away. Then, with a very sharp razor, slices should be removed from the rows of ovules

so as to lay bare the deeper layers of the nucellus. If the embryo sac is opened the contents may be lost, or, on the other hand, exceptionally favorable results may be obtained. Large ovaries may sometimes be cut transversely into thin slices, thereby exposing the ovules more perfectly than if the ends alone were cut off. In ovaries with axial placentation the axial tissue may often be split up into as many strands as there are rows of ovules and these rows then removed and fixed individually so that correct orientation of each for longitudinal sections is obtained, and a great economy of material effected. Solid fixation of the nuclei is not necessary for studies on the development of the embryosac, and even a little shrinkage (it is almost inevitable) may be tolerated. But if a study is to involve nuclear cytology, meiosis, gamete fusions, etc., then the same criteria must be applied as in studies on microsporogenesis or mitosis (p. 209).

*Sporangia* of bryophytes, pteridophytes and large microsporangia of gymnosperms should be cut open under the surface of the fixing fluid to facilitate immediate contact of meiotic cells and the fluid. Material not amenable to the orthodox methods should be abandoned for critical studies unless new methods can be worked out which will enable it to yield slides of a high standard of excellence.

### IX. Choice of the Fixing Fluid

It is necessary to select the class of fluid to be used on a given subject by a process of elimination, having in mind the character of the material and the type of result required. The material and its possibilities will determine (a) that the most difficult studies on nuclear and cytoplasmic structures are possible by reason of its easy adaptability or (b) that greater or less difficulties interfere and only more obvious features of structure can be studied. For this second class it is wasteful to apply delicate methods, and more or less deliberate scientific falsehoods are perpetrated when an attempt is made to interpret the results beyond the natural limitations imposed by the material. For the first class the most delicate methods and the most critical interpretations may be attempted, or if only the more obvious features are of interest then quicker, less fussy methods may be used and interpreted accordingly.

Under appropriate headings special suggestions are given for each great group of plants. Only the broader features are outlined here, in large part the angiosperms being used as a basis for the presentation. First, one must notice the amount of interference offered to the penetration of the fixing fluid. If after the preparation of the material has been completed (p. 213) thick pectic layers, cutinized, suberized or lignified



membranes interpose themselves between the fixing fluid and the cells to be studied, then only the most uncritical results can be expected, and such formulas as those of Carnoy, Farmer or Gilson (pp. 217, 218, 219) should be tried, for they are best fitted to overcome the resistance of the enveloping material. If the interference is less, and is limited to thin mucous layers, but a few layers of cells with only moderately heavy cellulose walls, a slight hairiness, etc., then (in addition to the fluids just named and with which more uncritical results are to be expected) more exacting studies may also be attempted and Navashin's, Němec's, Flemming's (strong) formulas, or Allen's modification of Bouin's fluid may be used. If the cells to be studied are, as a result of the preliminary manipulations, directly exposed to the fixing fluid, the exactness of action of the fluid is greatly increased. The results in these cases are far more under the control of the experimenter than in the others. It becomes possible to fix for a particular feature of the cell structure, a particular phase of mitosis. The student may take the more critical fluids and alter the proportions of the constituents to suit the demands of the occasion, especially with the two most adaptable, chrom-osmo-acetic and picro-formol-acetic with chromic acid. In doing this due regard must be given to the specific functions of each of the constituents as described in the general section (p. 9). It is remarkable that when the cells to be fixed are exposed directly almost all fluids function more perfectly than otherwise. Even quite violent fluids (as Gilson's) show unexpected values for demonstrating particular features of the cell. However, the writer would recommend that the investigator make every effort to apply one of the chrom-osmo-acetic group of formulas and to adjust it rigorously to the material and the problem, for he feels that these combinations are the most logical in organization and the most adaptable, and that the work of very many investigators (including those most skilled in both plant and animal cytology) has demonstrated that practically all cell structures (apart from mitochondria and a few other similar specialized cytoplasmic elements) can be better demonstrated by a well considered application of a chrom-osmo-acetic fluid than by any other so far devised. Having established by this method a certain hypothetical standard it is then advisable to try adaptations of other formulas, which often yield highly desirable short cuts to the demonstration of particular features. Delicate structures of higher plants (as developing trichomes, stripped epidermi from bulbs, etc.,) may be handled like delicate sporogenous tissues. It is notable that if the amount of vacuolization of the living protoplast is low and the fluid can act directly, formulas with very violent action (Carnoy, Farmer, Gilson, pp. 217, 218, 219) can be used without the usual great shrinkage; consequently these have had a

deserved popularity in work on filamentous fungi and on cells of similar structure, such as are occasionally found among algae or developmental stages of higher plants. They should always be avoided if possible when there are one or more prominent vacuoles in each cell.

Many of the fixing fluids used in plant technique are the same as those used in animal work (p. 552). Because of the resistance offered by the cellulose or indurated walls in plant tissues it has become customary to use formulas more notable for their penetrating power and the ability to preserve unshrunk the grosser cell elements than for the precision with which they maintain the finer nuclear or cytoplasmic details. It is urgently necessary to realize that a solution giving a slide of a good general aspect may preserve in a totally untruthful fashion one or many details of cell structure, so that in cytological work the fluids should be selected and modified for very specific uses, and general-purpose formulas should be distrusted for definitive studies. The following formulas are in some cases somewhat modified from their original form. None are necessarily in the best adjustment for any given piece of work, and none should be discarded for a specific purpose without a carefully planned series of experiments involving such changes in the properties of the constituents as their individual characteristics suggest, and also changes in the manner of immersion and the temperature of fixation (p. 564).

*Bouin's Solution* (p. 560). In its original form it is not suited to plant protoplasm.

*Bouin's Solution, Allen's Modification* (p. 561). This gives a fixation notable for the brilliancy of the staining with Heidenhain's hematoxylin, and is specially suited for the making of chromosome counts. It is not (in the present writer's opinion) suited for studies on prophase chromatin conditions. It is not suitable for use before Flemming's triple stain. It should act for ten minutes to a few hours, depending on the material.

*Carnoy's Fluid* (p. 558). Since this fixes by dehydration it is a fundamentally unsound combination for critical cytology; it usually produces extreme shrinkage, but may be adopted if it is necessary to penetrate hairy or cutinized surfaces and especially as a pretreatment before chromosmo-acetic mixtures, when it should be used for a short time varying from a minute or two down to but a momentary immersion. Such treatment is indicated when cuticularized or suberized surfaces, or masses of waxy or resinous hairs must be passed, and transfer to the effective fixing solution should be prompt. Farmer's fluid may be used in the same way.

*Chrom-acetic Solutions.* These solutions have good power of penetration, preserve the chromatin in its coarser aspects tolerably well, but fail to precipitate the karyolymph and some elements of the cytoplasm. Prophases are not well preserved, metaphases frequently are clumped

and the chromosomes contract longitudinally. While the fluids may be used for histological purposes, for general cell topography and for chromosome counting on easy material, they are not as good as the mixtures containing also osmic acid. *Weak chrom-acetic.* 10 per cent chromic acid, 2.5 c.c., 10 per cent acetic acid, 5 c.c., water to 100 c.c. *Medium chrom-acetic.* 10 per cent chromic acid, 7.0 c.c., 10 per cent acetic acid, 10 c.c., water to 100 c.c. *Strong chrom-acetic.* 10 per cent chromic acid, 1.0 c.c., 10 per cent acetic acid, 10 c.c., water to 100 c.c. The fluids should act for a few minutes on delicate specimens, from twelve to twenty-four hours on tough ones. Washing may be done for approximately equal periods with running or frequently changed water, or during dehydration with alcohols provided the material be kept in the dark. The weak solution is suited for filamentous algae, fungi, prothallia and objects a few cells thick, the medium solution for root tips and ovaries, the strong solution for woody material and tough leaves.

*Farmer's Fluids.* Absolute alcohol 6 pts., glacial acetic acid 1 pt., or a stronger formula, absolute alcohol 2 pts., glacial acetic acid 1 pt. The remarks and directions under Carnoy's fluid apply here as well.

*Chrom-osmo-acetic Fluids.* This group of formulas represents a few of the possible combinations of chromic, osmic and acetic acids. Mixtures involving these substances give by far the most truthful representation of most of the elements of the plant cell, and a formula of the Flemming type is to be adopted if at all possible in studies on chromatin behavior or the cytoplasmic mechanisms with the exception of mitochondria and a few other organs. It is best to keep the osmic acid for these formulas in a chromic-acid solution, for there it is more stable than in a simple aqueous solution. As, however, the addition of acetic acid or other organic adjuvants decreases the stability, it is best to make the final solutions up just before use. The formulas as given here are organized on the basis of a mixture of chromic and osmic acids. *Benda's Modification.* Chromic acid, 10 per cent aqueous, 3.1 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 12 c.c., glacial acetic acid, 6 drops, water 41.9 c.c. *Bonn Formula.* Chromic acid, 10 per cent aqueous, 0.33 c.c., acetic acid, 10 per cent aqueous, 3.0 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 0.62 c.c., water 6.27 c.c. *Weak Flemming.* Chromic acid, 10 per cent aqueous, 1.5 c.c., acetic acid, 10 per cent aqueous, 1.0 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 5.0 c.c., water 96.5 c.c. *Strong Flemming.* Chromic acid, 10 per cent aqueous, 3.1 c.c., osmic acid, 2 per cent in 2 per cent aqueous chromic, 12 c.c., 10 per cent acetic acid, 30 c.c., water 11.9 c.c. *Flemming for Smear Method and Root Tips (Taylor Modification).* Chromic acid, 10 per cent aqueous, 0.20 c.c., acetic acid, 10 per cent aqueous, 2.0 c.c., osmic

acid, 2 per cent in 2 per cent aqueous chromic, 1.50 c.c., water, 8.30 c.c., maltose 0.15 gm. This last formula has proved exceedingly valuable in studies on chromosome form and structure. It should be made up in small quantities as required, with the aid of a 1 c.c. pipette graduated to 100ths. The amount of maltose is subject to variation at the discretion of the experimenter, and seems to aid in keeping the chromosomes from clumping, in preventing satellites from fusing with the main chromosome body or segmenting constrictions from being obliterated. La Cour<sup>36</sup> has given a good résumé of modern fixing methods, including some effective new mixtures. That designated 2BE is representative and consists of chromic acid 10 per cent, aqueous 6 c.c., distilled water 134 c.c., potassium bichromate 1 gm., saponin 0.05 gm., acetic acid 10 per cent aqueous 5 c.c., osmic acid, 2 per cent in 2 per cent chromic acid, 15 c.c.; it is adapted to use on smears and small root tips. In general the weaker solutions are used on the more delicate filamentous plants, the stronger solutions on the more resistant ones. They should be allowed to act from a few minutes to twelve to twenty-four hours, and washed out as for the chrom-acetic series. All ordinary chromatin or cytoplasmic stains do well after these mixtures.

*Gilson's Fluid* (p. 557). Fixation is rapid, a few minutes usually sufficing if the objects are small.

*Helly's Fluid* (p. 558). It does very nicely for chromosome studies if the material is imbedded in celloidin rather than paraffin. Treat as after Zenker's fluid.

*Němec's Fluid*. Chromic acid, 1 per cent, 25 c.c.; formalin, 2 c.c. Use a freshly prepared solution and fix for about six hours. Then pour off and replace with a fresh portion, to act for a further eighteen hours. The time is to be reduced for delicate organisms.

This solution and its variants can be used in a rapid technique<sup>37</sup> by going direct to 75 per cent ethyl alcohol and by intergrades to absolute normal butyl alcohol, following the butyl alcohol technique into paraffin.

*Navashin's Fluid* (after Karpechenko). Chromic acid 10 per cent aqueous, 1.5 c.c., acetic acid, 10 per cent aqueous, 10 c.c., formalin 0.83 c.c., distilled water 23.67 c.c. See remarks under Němec's Fluid.

*Schaudinn's Fluid* (p. 533). This powerful mixture serves to fix on the slips spermatozooids, algal zoospores and flagellate types. It should be washed out with alcohol.

*Stromsten's Fluid*. Stock solution: chromic acid, 10 per cent aqueous, 16 c.c., acetic acid, 10 per cent aqueous, 100 c.c., water, 54 c.c. At the

<sup>36</sup> La Cour, L. *J. Roy. Micros. Soc.*, 51: 119, 1931.

<sup>37</sup> Randolph, L. F. *Stain Technology*, 10: 95, 1935.

moment of fixing add 2 parts of this to 1 part of formalin and immediately immerse the material. No washing is required and material may be preserved in the residual fluid, which changes color soon after mixing. Before imbedding it may, of course, be washed out in dehydration.

### X. Imbedding and Staining

The cytological material after fixation is generally followed by dehydration (p. 162, longer schedule), clearing through cedar oil, or better, xylol-alcohol changes (p. 163), and infiltration with paraffin (p. 179). Or, after dehydration the celloidin method may be followed (p. 180) using a slow and careful schedule without heat. Cutting is accomplished by the usual means, but especial care is needed to keep the knives in perfect condition so as not to break or dislodge the chromosomes or other cell structures unnecessarily. The thickness of the sections necessarily varies with the problem, but usually ranges from 8  $\mu$  to 12  $\mu$  for microsporogenesis studies, and 12  $\mu$  to 20  $\mu$  for embryosacs. The sections are affixed to the slips in serial order by the usual means (p. 183). Staining methods available are varied, Heidenhain's hematoxylin being the most dependable, but with a wide choice of aniline combinations (p. 187). The sections are usually mounted in damar or Canada balsam (p. 201). Minute objects not to be sectioned are handled in tubes by sedimentation, the reagents being supplied in succession. Filamentous algae and fungi are also frequently studied cytologically without sectioning (pp. 226, 236).

### XI. Cytoplasmic Inclusions

The methods of preserving tissues so as to retain various of the solid inclusions common in plant cells have been described (p. 159). Further treatment leading to their recognition may be briefly indicated here, but as the topics are more microchemical than cytological in their nature, reference should be made to strictly microchemical discussions for more complete information.

*Starch grains* freed from tissues (such as commercial starches) may be mixed with crystal or gentian violet and stained, the dye then poured off and the starch washed with saturated aqueous picric acid. The lamellae should be well demonstrated by this method. After picric acid the starch may be dried and mounted in balsam. The method may be adapted to sections, where safranin will also stain starch, as will thionin. For the simple recognition of starch aqueous iodine-potassium iodide is

the customary reagent, and should not be used too strong, as the dark color will obscure the lamellar structure of the grains.

*Protein* stored in granular form as food reserve in cells is usually preserved by the ordinary fixing methods. Lyons blue among other stains strongly affects such material. The more complex aleuron grains are readily fixed by alcoholic mercuric chloride or picric acid. The ground substance is satisfactorily stained with alcoholic eosin, when the globoid and the crystalloid stand out by contrast.

*Crystals* (rhapides, tabular and conglomerate or druse crystals) are generally calcium oxalate, more rarely of calcium sulphate or other substances. Barium chloride solution deposits barium sulphate about the calcium sulphate crystals but not about those of calcium oxalate (p. 158).

*Inulin* is in solution in the cells, but permanent preparations showing the sphaerocrystals in which it is deposited by alcohol are readily made (p. 158).

*Glycogen* is likewise in solution. It gives a brown color with iodine (iodine 0.1 gm., potassium iodide 0.3 gm., water 45 c.c.) which temporarily disappears on warming the preparation. The substance can be precipitated in the cell. Material is preserved (e.g., fungus hyphae) for twelve hours in alcohol and then for twelve hours in 10 per cent tannic acid. It must then be very quickly washed (with the aid of a centrifuge) and stained in iron chloride solution, which colors the glycogen black.

*Paramylum* is a type of stored carbohydrate frequently found in lower plants. The grains are apparently homogeneous or lamellate, rounded, rod-shaped, box-shaped or ring-shaped. They do not give the starch reaction with iodine, remain unaltered in 5 per cent potassium hydroxide, but swell readily and dissolve in a 6 per cent solution.

*Leucosin* is a stored reserve also found in lower plants. It appears to dissolve in water when the cells are treated with fixing agents, acids and alkalies.

*Fucosan* forms vacuolar bodies in the cytoplasm of Phaeophyceae, which are insoluble in water and do not stain with iodine. It reduces osmic acid and the older vacuoles blacken especially quickly. The vacuoles stain readily with methylene blue. The fucosan vacuoles are fixed by 25 per cent hydrochloric acid or sulphuric acid.

*Volutin* forms rounded bodies in bacteria, fungi, etc. These are readily fixed by alcohol, formaldehyde or picric acid. They stain readily with fuchsin and methyl violet, but resist eosin, and do not stain by Gram's method. They darken readily with iron-alum hematoxylin, but decolorize very readily on differentiation. Material strongly stained with carbol-fuchsin is treated with aqueous iodine-potassium iodide, when only the volutin retains the stain.

*Tannin* vacuoles are frequently found, especially in algae. They may be readily stained in the living state with methylene blue (1:500,000). The stain may be fixed with saturated aqueous picric acid in a few hours, rinsed, dehydrated, cleared through xylol, and mounted in balsam.

*Cystoliths* of calcium carbonate are occasionally found, and require especial care in preservation (p. 157). They readily dissolve in dilute acids.

*Sulphur* is frequently found as globules in a pure state on or in filaments of algae, bacteria or fungi growing in sulphurous waters. These globules are soluble in carbon bisulphide if the filaments are first killed, as by drying.

*Resin* masses are usually colored green in material preserved in solutions containing copper acetate (p. 159).

*Caoutchouc and latex* are treated under latex vessels (p. 159).

## XII. Fats<sup>38</sup>

Sections are made either with the freezing microtome or free-hand. Free-hand sections are better for all soft plant tissues, since freezing destroys delicate cells and may change the state of cell contents. Leaves, even small soft leaves, can be folded at the midrib, rolled gently between thumb and forefinger into a compact roll and sectioned free-hand. Small seeds and spores may be imbedded in paraffin. Put the seeds into a small porcelain dish or tiny paper box, pour hot (not boiling) paraffin over them, and arrange in any desired position with a hot needle. When cooled, cut the paraffin cake into pieces convenient for holding and section free-hand. Hard coated seeds are best sectioned with the freezing microtome.

Neutral fats in storage organs in plants are either in the form of semi-solid globules (solid in a few plants) in a matrix of protein or are in fluid state. Those having a high proportion of oleic acid are fluid. Fats in meristematic regions are present as small droplets which may be extremely minute and difficult to identify.

Two or more types of reactions are always made for the identification of substances in sections of tissue. The three most important reactions for fats in plants are: 1, solubility; 2, staining (with Sudan III or scarlet red); 3, saponification, and 4, myelin formation.

1. **Solubility.** Fats and fatty oils are insoluble in water, alkalies, and salts. They are soluble in ether, chloroform, acetone, absolute alcohol (boiling), xylol, balsam, and some other substances.

Put sections on a slip in a drop of water. Examine the cells carefully

<sup>38</sup> Section on "Fats" by Sophia H. Eckerson.

for drops having high refraction. Test their solubility in the fat solvents. Draw off the water with small strips of filter paper, adding 70 per cent alcohol at the other side of the cover glass. Then add a few drops of ether. Small droplets flow together and dissolve. Larger globules break up into small drops which flow together before going into solution. Test the solubility of the supposed fat drops in chloroform and in acetone. Lecithin, cholesterol, and the phytosterols are soluble in most of the fat solvents but are insoluble in cold acetone.

Ethereal oils and resins (also caoutchouc in latex plants) may be present in the tissues as glistening drops; or resins may be in solid masses, usually colored yellow to brown. These are soluble in the fat solvents but can be differentiated with a fair degree of accuracy by their greater solubility in certain substances. Ethereal oils are easily soluble in glacial acetic; fats and fatty oils are soluble with difficulty.

Add a drop of glacial acetic at edge of the cover glass. Ethereal oils dissolve; fats remain (ricinus oil is soluble). To other sections add a drop of chloral hydrate (5:2 in water). Caoutchouc drops swell greatly but do not dissolve (Molisch<sup>39</sup>); ethereal oils dissolve; fats remain. Resins are only slightly soluble.

Ethereal oils can be removed from the sections by microdistillation (Tunmann<sup>40</sup>) in a few minutes. Sections are placed on fine gauze or cheese-cloth held between two rings and placed over a small steam bath. The sections can then be re-examined and the remaining drops tested with concentrated  $H_2SO_4$ . Resin drops or masses freed from ethereal oils dissolve partially or completely. If small granules are formed they do not flow together. Fat drops break up into smaller drops which flow together.

## 2. Staining.

*Sudan III.* This dye is insoluble in water; soluble in alcohol, but more soluble in fats and oils. This fact is the basis of its use as a fat stain. When a solution in alcohol comes in contact with fats, the dye leaves the alcohol and dissolves in the fats, coloring them yellowish red. In order to act as solvents for the dye, fats must be in fluid or semi-solid state. In the case of solid fats, a slightly higher temperature, rendering them somewhat pasty, is usually effective.

A concentrated solution (approximately 0.5 per cent) of sudan III in 70 per cent alcohol filtered and stored in a tightly stoppered bottle keeps a year or more. When testing for fats care must be taken to prevent evaporation of the alcohol with consequent precipitation of the dye.

Mount sections on a slip in water and examine. Draw off the water by means of strips of filter paper, replacing with 70 per cent alcohol. Then

<sup>39</sup> Molisch, H., *Mikrochemie der Pflanzen*, Ed. 2, Jena, 1921.

<sup>40</sup> Tunmann, O., *Pflanzenmikrochemie*, Berl., 1913.



add a drop of the sudan III solution. After 15 to 20 minutes, draw off the dye, adding 70 per cent alcohol at the opposite side of the cover glass. Draw through, adding more alcohol to remove all of the excess dye. Then add a drop of glycerin or of levulose jelly (8 gm. levulose in 10 c.c. water) diluted with a drop of water to hasten the flow. Fats and fatty oils will be stained red; also suberin and cutin membranes, and ethereal oils and resins will be red if they are present. For permanent mounts levulose jelly is rather better than glycerin, since it clears better and hardens quickly.

*Sudan IV* (Scarlet red). This dye, like Sudan III, is insoluble in water, soluble in alcohol, and more soluble in fats, oils, and resins and in suberized and cutinized membranes. Since these dyes do not form salts (Michaelis<sup>41</sup>), they are ideal fat stains, especially for plant tissues where the cells contain many different substances.

A concentrated solution in 70 per cent alcohol, filtered and stored in a tightly stoppered bottle, keeps for many months. Ten to 15 c.c. for daily use should be kept in a 15 c.c. bottle having a ground-in pipette with rubber nipple. If the pipette is always replaced immediately after use, to prevent evaporation of the alcohol, the dye does not precipitate out.

When only minute droplets of fat are present in the cells, stain with scarlet red. After ten to fifteen minutes, remove all of the excess dye by drawing it off with filter paper, replacing with 70 per cent alcohol, until the filter paper shows no slightest trace of color. Then add a drop of concentrated  $H_2SO_4$ . The red droplets flow together becoming blue as the dye dissolves in the acid (Tunmann).

Michaelis' method is better since it does not involve the use of strong acid: After staining with scarlet red and removing all of the alcoholic dye, as before, add water, drawing off the alcohol. The scarlet red which was dissolved in the fat droplets is precipitated out in tiny clusters of red needle crystals.

Other dyes which are soluble in fat, oils, resins, and in suberin and cutin membranes are sometimes used advantageously. All are made up in concentrated solution in 70 per cent alcohol.

*Alkanin* (deep red) is a good fat stain. The chief objection is the long time required, four to sixteen hours. There seems to be a slow penetration into the cells as well as slow solution in the fats.

*Indophenol* as a free base stains fats blue. Its salts do not stain fats (Michaelis).

*Oil red A* (Nat. Aniline & Chem. Co.) stains fats yellowish red. It is a

<sup>41</sup> Michaelis, L., Fett. in Krause, R. Enzyklopädie der Mikroskopischen Technik, Ed. 3, Berlin, 1926, vol. 1.

rapid stain but the sections do not clear as well as after the use of Sudan III or IV.

*Dimethylamidoazobenzol* as a free base dissolves in fats giving them a clear yellow color, but it forms red salts with acids. The salts are not soluble in fats. As a fat stain it is satisfactory only with tissues containing neutral fats. In the case of acid tissues the cell contents are likely to be pink while the oil droplets may be faintly yellow or unstained.

*Osmic Acid.* Osmium tetroxide is reduced to black metallic osmium by oleic acid, olein, mixed fats containing oleic acid, ethereal oils, resins, lecithin, and tannins. These substances are, in turn, blackened or browned by the precipitated osmium. The saturated fats and fatty acids do not reduce osmic acid. Thus the reaction is of limited usefulness as a fat test.

Examine sections in water on a slide for highly refractive drops. Draw off some of the water, adding a drop of 1 per cent osmic acid. Note blackening. The nature of the blackened or browned substance must be determined by other means.

**3. Saponification.** This specific reaction should be made always in addition to the preliminary solubility and staining reactions. The best saponification fluid for the identification of fats in cells is made up of equal volumes of concentrated water, KOH and 20 per cent ammonium solution (Molisch), stored in a brown bottle. It is well to make up a fresh solution every six months, since it deteriorates on long standing.

Put sections on a slip in a few drops of the saponification fluid and cover, avoiding the inclusion of air-bubbles. Ring the cover glass with wax to prevent the formation of carbonates at the edge. Since from three to five days are required for complete saponification, it is best to keep the slide in a moist chamber. Observe the progress of saponification at intervals up to the fifth day, when it should be complete.

Usually in sixteen to twenty-four hours fine needle crystals of the potassium salt of the fatty acid appear at the periphery of some of the oil drops. Oil drops at the edge of the section show the beginning of saponification before those within the cells. After two to three days most of the fat drops will have the needle crystals at the periphery. A few drops may be completely saponified, leaving a group of the soap crystals. Some fat drops, without changing form, become doubly refractive, indicating that the fat is partially saponified. This type of partial saponification occurs more frequently if the strength of the saponification fluid is decreased, by aging or by dilution. By the fourth or the fifth day every fat drop should be completely saponified.

Tartaric acid and some alkaloids form crystals with potassium hydroxide but they are readily differentiated from the potassium-fatty acid crystals. The potassium-alkaloid crystals are formed quickly, usually

within 30 minutes; and they are in scattered clusters, not on the surface of droplets. Potassium tartrate does not form needle crystals.

**4. Myelin Formation.** This specific reaction is especially valuable for rapid identification of fat globules. Essentially it is partial saponification with the formation of doubly refractive drops.

Observe sections on a slide in 10 per cent ammonia or in 10 per cent KOH. Usually within 30 minutes the contour of fat drops changes and little protuberances appear, which may enlarge somewhat. Gradually the drops become doubly refractive.

The remarkable myelin forms frequently pictured occur with the fats of comparatively few plants but all fats and oils show the early change of the globules from spherical to an irregular form. (Sophia Eckerson).

### C. SPECIAL METHODS FOR PARTICULAR PLANT GROUPS

It was advantageous to treat the first portion of the present chapter from the viewpoint of one plant group. The angiosperms were selected as offering the widest range of methods, and the most familiar illustrations. Therefore it is not necessary to discuss them much further in this second portion. In the degree to which the other plant groups differ in size and texture special methods are needed, and these are suggested here, with references back to the first portion. The bacteria have been handled separately (Conn, Chap. III).

## XIII. Algae

In general the methods recommended in the more general sections for the preservation and preparation of filamentous specimens are applicable to algae, so that careful attention to the fixing and staining sections will be sufficient to give a working basis for the start of an algal study involving the development of a precise technique. Therefore little attempt will be made to present here a complete description of methods, rather emphasizing the special aspects of a few peculiar adaptations.

**1. For herbarium specimens** and general morphological studies many filamentous types are preserved quite sufficiently well simply by drying on clean glass slips or freshly split mica. These preparations, if made of marine organisms at the seashore, may remain moist, due to the hygroscopic properties of the salt, and so should be dried in the sun or a desiccator and kept carefully wrapped in a moisture-proof container until they can be filed under dry atmospheric conditions. When desired for use they may then be moistened with water, covered and studied with ease and without disturbing the specimen, and dried again for storage.

Care should be exercised in reporting cell measurements based on such material. It may be better to moisten with carbol-glycerin or lactic-glycerin (p. 198), but this must be well washed off if the material is to be again dried. (For the handling of herbarium material dried on paper see p. 158.) Algae which adhere as close films to the paper are sometimes difficult to remove. With a keen knife cut lightly through the surface of the paper around the margin of the piece needed. Then cover with a drop of water, let it stand for a time, and finally gently scrape loose the desired portion of alga with scalpel and needle, transferring to a drop of lactic-glycerin on a clean slip, where further dissection may be accomplished and the drop covered for study or preservation. Eosin may be used to stain such material before placing in lactic-glycerin.

2. **Properly fixed algal material** is most generally secured by a suitable modification of Flemming's fluid applied effectively, since this fluid may be expected to give the best results. It is usually well to start with a weak formula, at least on the filamentous types. Marine forms are, of course, fixed in solutions made up with sea water. Chrom-acetic mixtures do well for general habit and grosser cell structure, while formalin-acetic-alcohol and Keefe's formula are good for preserving habit material (p. 157). It is only common sense to avoid drying material on the slide, either before or after fixing, for any but crude morphological studies. However, drying may be all right for observations on flagellae (p. 205), and does no great harm for observations on the position of nucleus and pyrenoid in small unicells, but cannot serve for observations on chromatophore structure, since the chromatophores readily shrink. The fixed specimens of filamentous or unicellular types are washed, stained carefully in Heidenhain's hematoxylin, safranin or magdala red, and counterstained if desired with erythrosin, orange G, aniline blue, cyanin or light green (p. 187). Then they are dehydrated (pp. 12, 26), cleared (p. 164), infiltrated in balsam (p. 202) or Venetian turpentine (p. 203) and mounted. The filamentous types may also be imbedded and sectioned for nuclear details, the proceeding being gradual (p. 163). The mucus so often present about algae frequently interferes with infiltration and imbedding. Dilute aqueous methylene blue will stain the cell walls of algae, particularly fresh Phaeophyceae, very readily. Mats of filaments (as *Vaucheria*) can usually be best prepared undisturbed and later teased or cut apart just before mounting, and crusts of filamentous or unicellular types or sticks or stones can be scraped off and dissociated likewise just before mounting. (For handling sporelings or species cultured on slides see p. 235.)

3. **Epiphytic and endophytic types**, if small, are best fixed, stained, and mounted in situ, or only removed just before mounting. For teach-

ing purposes it is often well to mount on a slip both light and dark stained samples, since chromatophores show best at quite a different degree of differentiation from that suitable for nuclei and pyrenoids.

4. *Myxophyceae* when collected may be badly mixed with other forms. *Oscillatoria* and hormogonia of other genera will creep out from such a mass if it is placed on a wet slip in a damp chamber, and will spread over it, where they may be dried, or fixed in some cases by inverting the slip flatly on the surface of a suitable fixing fluid. Dried mounts preserve the cell organization more effectively here than with most algae, but are quite unreliable from the cytological standpoint. Many species of the *Stigonemaceae* show splendid intercellular connections. Accurate fixation through the sheath frequently present is probably impracticable with present methods; indeed, critical inspection of the results of cytological studies on *Myxophyceae* suggests that little, if any, adequate fixation has been accomplished in the group. Staining with Delafield's hematoxylin is reported to give a polychrome result, some granules being blue and others pink in tendency. While the writer has seen no such specificity, it does give a good general stain. Heidenhain's hematoxylin followed by erythrosin gives good contrast. Safranin stains mature spores well and can be followed by aniline blue, light green, crystal violet, or Delafield's hematoxylin. Methyl green stains young heterocysts readily.

5. *Chlorophyceae* of the motile groups are handled in general like independent flagellate protozoa (p. 522). Fixation is excellent after Flemming's fluid, but hot sublimate-acetic may serve well for Volvocales. The sedimentation method of handling, even to the use of a centrifuge, is preferably used for passing the organisms through the fluids. Dehydration by the concentration of glycerin is easiest and best. The sample should be agitated as little as possible to avoid clumping and the breaking of the flagellae. They may be stained and mounted entire as strewn mounts, using the balsam or the Venetian turpentine infiltration methods, or they may be sectioned after imbedding in paraffin.

*Desmids*, if of the filamentous genera, are handled like *Spirogyra*. Unicellular forms, when in great abundance, are handled by sedimentation. If rare they are picked out with a fine pipette, and placed in a small drop of water on a slip which is inverted over the mouth of an osmic acid bottle, where the vapor fixes and darkens the cell. This is flooded with 10 per cent glycerin that is allowed to concentrate, and later replaced, if desired, with glycerin jelly for mounting. The sheath of desmids, both filamentous and unicellular, can be effectively stained by a method proposed by the writer.<sup>42</sup> "Fresh living material is placed in a

<sup>42</sup> Taylor, W. R. *Trans. Am. Micros. Soc.*, 40: 94, 1921.

.05 per cent aqueous solution of methylene blue for forty-five to sixty seconds. It is then removed, rinsed in distilled water and placed in a  $\frac{1}{10}$  saturated aqueous solution of picric acid. This serves to fix the stain and brings out in a most striking manner the striations in the sheath. The material may be examined in the picric acid solution, or removed after a minute or two to water. Preparations are best used soon after staining, as the sheath begins to disintegrate after a few hours." This is an especially favorable method for Placodermæ, such as *Hyalotheca*, whose sheath shows very well the radiations resulting from its method of formation.

*Chlorophyceae* of the larger filamentous groups are handled after the general system for filamentous organisms, being especially careful to avoid shrinkage of the protoplast. Chamberlain<sup>43</sup> recommends keeping the chromic acid up to 1 per cent and increasing the acetic acid until shrinkage is overcome, as he finds that algae require more acetic acid than do higher plants. Flemming's fluids are generally better than chrom-acetic, though differentiation of stains may be somewhat more difficult. *Spirogyra* gives especially splendid mounts when carefully handled. The fixed filaments are gently washed, stained in Heidenhain's hematoxylin with care to avoid overbleaching in differentiating, well washed and placed in 2.5 to 5 per cent glycerin. Here they are dehydrated by concentration, the process occupying two to three days to a week. It should not be unduly rushed, and must be complete. Nucleolus, chromosomes and pyrenoids should be black, chromatophores steel gray, and general cytoplasm pearl gray. The contour of the chromatophore margin should show the characteristics of the species used. No counterstain should be necessary. After dehydration the balsam infiltration method (p. 202) should be followed. Conjugating material is somewhat more sensitive than sterile filaments, especially to the xylol-balsam change. Filaments with zygospores are most effectively stained with safranin-aniline blue, the spores being bright red (p. 189). Massive Chlorophyceae (Codiaceae) offer no special problem, for pieces of tissue may be handled like pieces of soft tissue from higher plants. If calcareous they may be hard to fix, and must be decalcified (p. 160).

6. **Charophyceae** are generally so sturdy that they should be handled like delicate parts of flowering plants for imbedding and sectioning. Care should be used in orienting the material for cutting, since from inappropriate angles the sections do not show the geometrical regularity of division planes which characterize the organs of the group. Apical cells are frequently needed for demonstration. Tips of main axes from sturdy types should be selected and the branches and leaves trimmed close to the tip. Fixation in Flemming followed by imbedding, sectioning

<sup>43</sup> Chamberlain, C. J. *Methods in Plant Histology*, Chicago, 1924, p. 176.

and staining in safranin and Delafield's hematoxylin is best. In addition to the apical cell these should show young stages of leaves, branches, and on fertile plants, of reproductive organs. For older stages of reproductive organs cut off branches of appropriate age and imbed individually. Sections of the branch are best cut longitudinally and axially through the reproductive organs. The branch may show a range of developmental stages. Mature antheridia may be punctured and stained in toto, and then dissected apart when ready to mount. Mature oögonia are extremely hard to cut. For habit mounts the more slender species are to be preferred, and they may be handled like filamentous algae, mounting under sufficient pressure to flatten out the specimen. Safranin and aniline blue will generally give a brilliant stain.

7. **Xanthophyceae** are not well enough known or widely enough studied to have developed special technique. In general they are handled like Chlorophyceae of similar form. The wall seems to be often more resistant to fixing and staining fluids. The peculiar wall structure, each cell having two overlapping portions, equal in size or like a lid on a cylinder, is emphasized by swelling with strong caustic potash and staining with Congo red, or by separating the portions by treatment with 30 per cent aqueous chromic acid, cold or with the aid of heat.

8. **Bacillariophyceae (Diatoms)** because of their peculiar silicified cell wall offer special problems in treatment for systematic study. Often the living cell content is a positive disadvantage, and must be removed before the species can be studied. If the living cell contents are to be observed they are prepared like desmids or other unicellular Chlorophyceae. Care is needed in destaining, for penetration of fluids through the wall is irregular. Material (after hematoxylin) should be removed from the iron-alum a little before it seems completely ready, so that it will not become unduly destained before the iron-alum can be washed out. The material should be dehydrated through glycerin and infiltrated with balsam. If the diatoms are to be studied from the systematic standpoint it is generally the silicified wall alone that is needed. A great number of methods of cleaning material have been devised, some of limited and others of wide application. For recent or living material two general systems will suffice.

The mass should be allowed to settle and the supernatant water poured off. An equal volume of concentrated hydrochloric acid is then poured on, mixed, and gently boiled. After a few minutes action will have been completed and the flask or test-tube is allowed to stand and the material to settle. A brief washing in 2 or 3 changes of water follows, and then the water is poured off and an equal volume of nitric acid poured on and similarly boiled, followed in turn by washing and

boiling in sulphuric acid. This last will usually cause darkening of the material through carbonization of any remaining organic matter. To clear this up crystals of potassium chlorate should be cautiously added one at a time to the hot acid, protecting carefully one's eyes and neighbors. The use of a chemical hood through all this process is helpful because of the unpleasant fumes. Each crystal of potassium chlorate will probably cause a small explosion and the formation of chlorine gas, which with the oxygen will effect bleaching and solution of the remaining organic matter. If a large quantity of material is being handled it may be necessary to repeat each stage of the process before passing on to the next.

In many cases it is sufficient to prepare a mixture of two parts of hydrochloric acid and one part of nitric acid, and to boil the sample of diatomaceous material in this for a few hours. The solution should be decanted and replaced with a fresh portion if there is an excessive amount of organic matter present, and loss by evaporation should likewise be replaced. The final washings must be thorough. The diatoms are then separated from the sand by fractional sedimentation, the coarse sand going down first, then coarser grit and large diatoms, and finally small diatoms and fine clay particles. It is possible practically to free any desired sample from extraneous matter by judicious and repeated sedimentation. Or, the sample may be placed in a small jar with a large quantity of water and the jar revolved between the hands, when the material will rise in the center of the jar and the particles become distributed according to their relative densities. After washing, material is best stored in small bottles with ample 50 per cent alcohol. In mounting it is necessary to use absolutely chemically clean cover glasses (pp. 21, 77), if the job is to be neat and the distribution of the frustules uniform. It is best to let the material in the bottle settle and replace the alcohol with distilled water. Shake and put a drop of material on each cover, where it should spread out evenly. After the diatoms have settled in the bottle replace the water again with alcohol for storage. Allow the covers to dry thoroughly without disturbing them, and then heat to drive off any water in the frustules or their pores. These are spoken of as strewn mounts. Add a drop of thin xylol balsam and heat. Then invert on a drop of balsam on a slip and warm until firm. Bubbles of gas formed by heating the balsam solvent will disappear in time, as will small air bubbles. The use of special highly refractive mounting materials recommended for diatoms is rather a special topic and reference may be made to older works on microscopy if difficulties are encountered. Many species show their more delicate markings best if mounted dry. "Cells" should be prepared by making several superposed rings of cement (p. 199)



on a clean slip. Covers with dried frustules are prepared as above, and inverted while quite warm so that their edges lie on the cement rings. Press each cover slightly into the ring so that it adheres. Let it cool and give a coat of rather thick cement. If well pressed into the ring no difficulty should be met from cement running under the cover.

*Selected diatoms* are generally isolated from a mass collection and mounted separately for reference. Strewn covers are prepared as usual and fastened, material up, on slips. A microscope equipped with a mechanical finger is used. A ring above the objective (about 8 to 16 mm. focal length) bears a lever or rod which can be raised and lowered by a screw, and pushed toward or from the optical axis through a smooth-fitting sleeve. A fine glass filament is cemented with wax at the tip of the rod. The free tip of this is moistened, usually with a very faint trace of oil or grease to make it slightly sticky. It is then adjusted to the center of the field of view and raised up out of focus by the screw. The objective is focussed on the slide and the diatom desired is selected. Then the filament of glass is lowered into contact with the diatom, which should, with a little manipulation, adhere lightly to it. With the coarse adjustment the objective and specimen are raised together under observation, and the strewn mount removed from the stage. Another slip should be ready bearing a cover in the center of which is a thin smear of gelatin dissolved in glacial acetic acid. This is slipped into place and the diatom lowered into contact, still under observation. By gently breathing on the slide, moisture causes the diatom to stick to the gelatin. On drying, the cover may be mounted as usual without disturbing the specimen, and its position marked by spinning rings of colored ink or cement about it as a center. By manipulation of the mechanical finger diatoms may be placed in any desired position, to show face, girdle or end views, and collections may be selected and arranged on a single slip, to several hundred in number, to exemplify the flora of a given sample, to give test plates with forms suitable for testing a wide range of microscope objectives, or even only to demonstrate the marvelous beauty of the diatoms themselves.

Diatoms are not usually abundant in highly calcareous collections, but on tropical shores are often admixed with a predominating bulk of foraminifera, calcareous sand and shells. Consequently a quantity of the original material may be greatly concentrated by gradual addition of hydrochloric acid to the sample till effervescence ceases, followed by sedimentation or centrifuging and the usual treatment. Plankton types and many bottom forms, especially of the tropics, are often so delicate that the severe first schedule offered above will destroy the markings or even the entire frustules. For these the sulphuric acid may best be

omitted, and the other treatments curtailed or performed with weaker acids. It may be possible to do no more than dry the sample on a slip without removal of the organic matter. Many coarse types are adequately prepared if a sample of the original material is spread on a cover, which is then placed material side up on a silver plate and this heated dull red or until the organic matter is burned away. This can generally be accomplished without softening the cover, which is mounted as usual.

*Fossil diatoms* in diatomaceous earths or soft diatomaceous rocks are generally free from much organic or calcareous matter. The sample is broken to fine granules and boiled in hydrochloric acid until the frustules may be separated by shaking. These are then separated by fractional sedimentation. Usually there is a great proportion of broken shells and these come away with the small species. Diatoms in marl are accompanied by clay and may be difficult to separate from extraneous matter. Diatoms in harder rocks are best shown by grinding thin sections after the fashion of other fossil samples (p. 174).

9. *Phaeophyceae* in general offer few peculiarities requiring special instructions. In general they can be handled like green algae of similar texture and form. The walls of filamentous types (as some species of *Ectocarpus*) are often quite horny in texture when imbedded and do not cut cleanly. They are best studied as whole amounts if possible, and are well stained in the fresh state by methylene blue. *Cladostephus* and its relatives show splendid asters at the division stages of the apical cell, and longitudinal sections of selected apices give good material for their study (p. 206). The soft, gelatinous walls of the fleshy browns swell and shrink greatly as they are wet or dry, and so material imbedded for sectioning in paraffin or celloidin generally becomes much distorted and sections readily work loose from the slides. The freezing method (p. 178) is advantageous with such types. In some species with dilute jelly between the cells or filaments prolonged preservation in formaldehyde (which probably becomes quite acid) dissolves the jelly so that the specimens imbed and cut perfectly in celloidin. Especially fine preparations exhibiting the structure and reproduction of the Chordariaceae may thus be made from *Castagnea* that has been stored for a couple of years. *Fucus* as the commonest laboratory type must frequently be attacked for the preparation of routine teaching slides. Flemming's fluid is the best fixing agent, and the material, in vigorous health, should be cut into the small pieces that are to be sectioned before fixation is attempted, in order that the gelatinous walls and the slime may interfere as little as possible with its action. For the growing tip the apices are cut from sterile branches as pieces 2 or 3 mm. square, fixed and sectioned in paraffin in the plane of the branch. The structure of the branch is

best shown in fresh transverse sections mounted in water. For the reproductive organs it is best to cut the receptacles lengthwise and then cut out small blocks with a few conceptacles, fix, imbed and cut in paraffin as transverse sections, selecting as best those that pass through the pore of the conceptacle. Heidenhain's hematoxylin followed by orange G or erythrosin gives the best stain, but Delafield's hematoxylin is good enough for material not Flemming-fixed. Eggs of *Fucus* may be fertilized and fixed at chosen intervals after scraping from the dish containing them, and to which they become more or less attached. By imbedding in paraffin sections may be secured showing the entrance of the sperm, fertilization and the first mitosis with the asters and other characteristic features. Sporelings of *Fucus* are easily mounted and instructive, and sporelings of other Phaeophyceae (especially Laminariaceae) are similarly valuable, prepared by the general method (p. 235).

10. **Rhodophyceae** offer considerable difficulty from the standpoint of technical practice. Unicellular types (as *Porphyridium*) are handled as smear preparations on clean covers. Filamentous types are fixed in masses and handled like the filamentous greens. Flemming's fluid is by far the best fixing agent. Some form of the weak formula should be used, and not allowed to act very long: a few minutes to an hour or so. The filaments disintegrate rather readily in dilute acids. It is best to wash while dehydrating slowly through the alcohols, using close stages for short periods and keeping the container in a dark place. Imbedding and sectioning, or staining and infiltration with balsam, follow the usual methods for filamentous Chlorophyceae. The large coenocytes of some genera (*Griffithsia*) call for great care in clearing. Safranin followed by aniline blue gives excellent contrast. Nuclei will generally be purple, chromatophores light blue and cell walls pink. Heidenhain's hematoxylin is dependable for accurate details. For morphological studies chrom-acetic or formol-acetic-alcohol is suitable, followed by preservation in 70 per cent alcohol. Fixation and preservation in formaldehyde is not recommended for filamentous types, since these tend to disarticulate as the formaldehyde becomes more acid. Fleishy species are best sectioned by the freezing method (p. 178), or imbedding in soap (p. 179). Calcareous species are very difficult from the cytological standpoint. Probably the best results are to be secured by fixing such genera as *Melobesia* and *Corallina* in a relatively large volume of Flemming's fluid and removing the CO<sub>2</sub> rapidly by the aid of a vacuum pump, adding additional chrom-acetic solution as the original becomes exhausted. Massive types such as *Lithothamnion* and *Goniolithon* are probably impracticable cytologically. For histological and morphological studies of these genera chrom-acetic decalcification and fixation is ade-

quate, and imbedding in paraffin best for cutting. As there is little tissue differentiation Delafield's hematoxylin alone is a good stain. In addition to these customary ways of preparing material it is often desirable to swell up developing procarps and cystocarps and to cause the cells to stand apart from each other. This is especially effective on *Griffithsia* (Lewis). Living material is fixed with chrom-acetic or with aqueous iodine and placed in 10 per cent glycerin. Here the walls of the procarps and cystocarps gelatinize and swell, so that the intercellular connections may be readily recognized. A trace of eosin in the glycerin will help by tinting the specimen. To trace the development of the cystocarp in those Cryptonemiales with long oöblastema filaments is not so difficult if the species preserve a distinctly filamentous structure and are soft. In the firmer types it is often difficult. *Agardhiella* will serve as a good example for demonstrations, being widely available. Select sturdy tips and split them lengthwise with a sharp razor, being careful to go exactly through the apex for best results. Lay both halves split side down upon the cover glass, and then invert upon a slip and add water from the margin. The carpogenic branches and auxiliary cells with trichogynes and oöblastema filaments can be seen upon the big cells of the medulla wall, having slightly denser contents which can be tinted with eosin.

11. **Sporelings and epiphytes** are effectively cultured on slides and stained and mounted in position. Smear mounts, bacterial zoöglea spreads, and other fungal mounts involving parts of, or entire, colonies, are familiar in method (pp. 135, 165), but for unicellular or attached filamentous algae, early stages of bryophyte or algal sporelings, etc., it is convenient to fix, stain and mount on the original substratum. The slips used should be absolutely clean. If the organisms seem to have difficulty in making firm attachment to the slips these should be etched on one side with hydrofluoric acid, ground with a sand blast, or given a matte grain by rubbing together with emery powder. When handling positively phototropic organisms (such as zoöspores from *Stigeoclonium*, *Ulothrix*, *Chaetophora*, *Ulva*, *Ulothrix* or *Oedogonium*) the slips should be placed in the container with the material, and between it and the light source. For spores of Rhodophyceae, or other non-motile spores, and eggs of the Fucaceae, the slips should be distributed over the bottom of the container. In any case as soon as the desired material has become attached, the slides should be removed to a clean container with fresh (preferably filtered) water, and handled by methods appropriate to the particular organisms under observation. At convenient intervals slides may be removed, placed in staining wells of fixing fluid and handled throughout as in the smear method (p. 165). Often highly interesting material (like *Coleochaete*) will form a green coating on the

walls of aquaria, and if slides are placed in contact with the walls a good growth will appear on them equally readily. Instructive mounts of small diatoms are a frequent by-product of this method. The fixing fluids and stains to be used are those ordinarily adaptable to whole mounts of filamentous algae (p. 227). For sporelings of Fucales simple dilute Delafield's hematoxylin seems best.

#### XIV. Fungi

The fungi are handled similarly to algae in many ways, for both are unicellular to filamentous types of organisms which attain rigidity and bulk in more complex groups by lateral association and specialization of the filaments. But the cell wall is of different material and texture, and chromatophores are absent. The fungi are, furthermore, often parasitic, and the nature of the host's tissues may determine the character of the technique even more than the parasite. Minute forms, if in great quantity, may be handled in bulk through fixing, staining and dehydrating. Yeast, for instance, may be managed in this way. Cultures of small forms, including bacteria (see Chap. III) and germinating spores may be spread on slips, dried, fixed by passing a few times quickly through a flame, and then stained. Or they (cultures, or spores) may be spread on slips which have been wiped with a little Mayer's albumen and are allowed nearly to dry, and then inverted flatly upon the fixing fluid, which may conveniently be in a Petri dish with some fragments of glass rod to keep the slides from coming in contact with the bottom. After fixation they may be washed and treated as sections. A method advocated by Bachmann<sup>44</sup> may give good service:

Prepare some thoroughly clean sterile slips. Onto one pour the culture medium (with gelatin or agar base) inoculated with a suspension of the organisms to be investigated. Place another slip upon the first and draw apart, so as to leave quite a thin film. The slides should then be incubated under sterile conditions in a damp chamber (as a Petri dish with wet filter paper on top and bottom). When the colonies are ready the slides may be fixed and handled precisely as if bearing smears or sections. For yeast she recommends potato broth agar, which, like all media for this method, must be cleared. Heidenhain's hematoxylin followed by a counterstain (if desired) and mounting in balsam is usual. Or yeast may be cultured in fluid and killed in bulk in a saturated aqueous solution of corrosive sublimate, where it may be left twelve hours, following with thorough washing in water, 30 per cent and 70 per cent alcohol and finally absolute methyl alcohol. A drop of a sus-

<sup>44</sup> Bachmann, F. M. *Amer. J. Botany*, 5: 32, 1918.

pension of the cells should be placed on a coverglass and allowed nearly to dry. The cover is then flooded with water, and the yeast allowed to settle. Then the water is drained away and the cover dried. After thorough drying, which should cause the yeast to adhere, the cover may be wet and stained with the combined fuchsin-methyl green mixture and mounted in damar balsam,<sup>45</sup> or Flemming's solution may be used, followed by Heidenhain's hematoxylin, then glycerin dehydration (p. 163) and infiltration in balsam (pp. 202, 557).

Many species of fungi, especially from culture on solid media, are in the form of a loose mass of mycelium. For direct examination it is usually necessary to remove the air from between the filaments in mounting, and this may be accomplished by flooding the material on the slide with 70 per cent alcohol. The material may be mounted for examination in 3 per cent acetic acid, 3 per cent potassium hydroxide or weak chloral hydrate. The potash is especially good if the sample has been dried. Aqueous media of a low refractive index are best, not lactic-glycerin and the like. For general fixation absolute alcohol, saturated corrosive sublimate with 1 per cent acetic acid in 95 per cent alcohol, or Gilson's fluid are to be recommended. Duggar<sup>46</sup> recommends for fungi a modified Gilson's fluid with the following composition:

Alcohol, 95 per cent, 30 c.c., distilled water 270 c.c., glacial acetic acid 2 c.c., nitric acid 5 c.c., mercuric chloride 10 gm. The usual procedure after a mercurial fluid should be followed (p. 11).

He also gives a special Flemming's type fluid: 10 per cent chromic acid 1.5 c.c., 10 per cent acetic acid 1.0 c.c., 2 per cent osmic acid in 2 per cent chromic acid 5 c.c., distilled water 37.5 c.c. He prefers to bleach the material in bulk in 95 per cent alcohol, 75 c.c., hydrogen peroxide 25 c.c. before imbedding.

Material of fungi is essentially exceedingly delicate, at least in the filamentous types, and offers considerable difficulty in handling through the alcohol changes and in imbedding. For transferring Duggar recommends little dipper-shaped wire gauze ladles, transferring the ladle with its contents from stage to stage without disturbing it. Material to be examined on the slide without extended treatment may be stained most readily with 0.5 per cent aqueous eosin, or with alum-eosin (0.5 per cent of each). Filaments hard to stain take most readily to Ziehl's carbol-fuchsin as prepared for bacteria. For material sectioned for histological or cytological studies the ordinary methods may be tried. Stages showing brightly colored spores (*Xylaria* ascospores, *Puccinia* teliospores) may be satisfactorily differentiated merely by the use of such a

<sup>45</sup> Chamberlain, C. J. *Methods in Plant Histology*, Chicago, 1924, p. 196.

<sup>46</sup> Duggar, B. M. *Fungous Diseases of Plants*. Boston, 1909.

counterstain as light green or Delafield's hematoxylin. *Xylaria* and other similarly tough forms may sometimes be cut satisfactorily unimbedded like stems of vascular plants. Apothecia of *Peziza* and genera of similar texture, including some lichens, may be broken up into small pieces, stained in bulk in aqueous eosin, rinsed, transferred to 2 per cent acetic acid and there teased out to show the developmental stages. Or the small pieces may be stained in eosin, dehydrated and cleared with clove oil, and teased out before mounting in balsam. For sectioning, old apothecia may be selected, imbedded in paraffin and sectioned rather thick, when the spores take safranin stain well, and light green or Delafield's hematoxylin may be used as counterstains. For the maturation divisions, including demonstrations of centrosomes and intranuclear mitosis, exceedingly small specimens must be used, and the sections cut thin and stained with Heidenhain's hematoxylin.

The differentiation of fungus mycelium from host tissue in the case of pathological infestation generally offers difficulty. The usual histological combinations may be tried, and may give satisfactory results, but frequently fail.

Vaughan<sup>47</sup> recommends the following procedure: A stain combination known as Pianese IIIb is prepared by mixing malachite green 0.50 gm., acid fuchsin 0.10 gm., martius yellow 0.01 gm., distilled water 150.0 c.c., 95 per cent ethyl alcohol 50.0 c.c. The tissue sections are washed in water or alcohol and stained in the mixture fifteen to forty-five minutes. The excess stain is removed with water and decolorized to differentiation with 95 per cent alcohol containing a few drops of hydrochloric acid. The material is then cleared with carbol-turpentine (carbol-xylol would do, probably) washed with xylol and mounted in balsam. He has also modified the method for the staining of germinating spores on the epidermis of infected plants. The leaf is prepared, a drop of spore suspension placed on the desired region and the leaf kept under suitable cultural conditions. At the proper time the test area is cut out, fixed for twenty-four to thirty-six hours in equal parts of glacial acetic acid and 95 per cent alcohol, washed in 50 to 70 per cent alcohol, stained for fifteen to thirty minutes in Pianese IIIb, washed in water for two minutes, very hastily run through acid alcohol, dehydrated and cleared as above.

Cartwright<sup>48</sup> recommends for mycelia in woody tissue that the sections be briefly stained in 1 per cent aqueous safranin and then rinsed. The counterstain consists of a mixture of 25 parts of saturated aqueous anilin blue solution and 100 parts of saturated aqueous picric acid, which is applied to the slides and these then gently steamed over a flame.

<sup>47</sup> Vaughan, R. E. *Ann. Missouri Bot. Gard.*, 1: 241, 1914.

<sup>48</sup> Cartwright, K. *St. G. Annals Bot.*, 43: 412, 1929.

Washing, dehydration in absolute alcohol, clearing and mounting in balsam should give slides with red wood and blue hyphae.

The mycorrhiza found in *Corallorrhiza* rhizomes and in the roots of epiphytic orchids frequently gives some difficulty in staining. The writer has had best success after fixing in chrom-acetic, and staining sliding microtome sections heavily with safranin, destaining, and counterstaining in succession with light green and orange G. The older mycelium and host nuclei should be deep scarlet, the younger pinkish or green, the host walls yellowish or if lignified, red. Cohen and Doak<sup>49</sup> for endotrophic mycorrhizas recommend an elaborate technique designed to retain the protoplasmic contents of the hyphae in good cytological condition. The fixing fluid consists of 5 per cent chromic sulphate in 4 per cent formaldehyde, which solution is saturated with either picric or salicylic acids. It is allowed to act for forty-eight hours and the specimens dehydrated by the butyl alcohol technique. The stain is applied, after hydration, to sections cut from paraffin at 12 $\mu$  thickness, and consists of 3 per cent acetic acid saturated with orseillin BB, and is allowed to act for thirty minutes. After rinsing in water the slides are dehydrated by a short ethyl alcohol series and stained in 1 per cent crystal violet in clove oil, washed with xylol and mounted in balsam.

Lichens give considerable difficulty in preparing sections of imbedded material, and may best be studied from sections of fresh or fluid preserved material cut unimbedded, though paraffin imbedding may be tried and will at times succeed perfectly. Heidenhain's hematoxylin followed by erythrosin, or cyanin followed by erythrosin make good stains.

Myxomycetes in the plasmodial state may be fixed excellently with Flemming's fluid, and if the plasmodium has been induced to climb onto a slip the flowing mass may be fixed as a whole mount. Imbedding in paraffin is all right if the material does not have to be kept with the substratum after fixing, but if it surrounds leaves of grass or decayed sticks it is better to use celloidin. The stages in the formation of the sporangia and the segmentation into spores and capillitium require very thin sections, and Heidenhain's hematoxylin or crystal violet by the Newton or Smith methods are the only stains precise enough to use for nuclear features. In fact, throughout the fungi, these stains are the only ones generally applicable to the study of nuclear details because of the very small size of the structures involved.

*Spores of fungi and other plants* may be caused to adhere to the slips upon which they are to be studied if these are smeared with a film of Mayer's albumen before the spores are spread on the surface. The slide should be inverted upon a suitable fixing fluid, or dried and

<sup>49</sup> Cohen, I., and Doak, K. D. *Stain Technology*, 10: 25, 1935.



heated, or inverted upon alcohol, to coagulate the albumen. After this, the technique to be followed is similar to that adopted for bacteria (p. 134) or smears of pollen grains (p. 165). Most often they are simply mixed with balsam or glycerin jelly and covered.

### XV. Bryophytes

The Bryophyta present, in general, few special problems. Preservation for general morphological purposes may be effected in Keefe's fluid, or in formol-acetic-alcohol, or formalin with cupric acetate (p. 159). Habit mounts of whole plants, and morphological studies of thallus form, leaf form and cell arrangement, spores and elaters, peristomes, etc., are most readily mounted unstained in glycerin jelly or in a viscous medium designed to minimize shrinkage (see p. 199). As they often make thick mounts the use of melted resin-lanolin for sealing is recommended (p. 199). In no group of plants is the use of free-hand sections more essential to the quick determination of species or interpretation of anatomical features (p. 176). Peristomes, calyptras, etc., for whole mounts in balsam should be dissected out and dried between two slips under light pressure. It is well to moisten with xylol before adding balsam to drive out air, which it may be difficult in any case to remove from the capsule and annulus cells, though boiling in balsam may help. Cytological studies can be best made on material fixed with Flemming's fluid, or the special fluids for mitochondria, etc. (p. 217). The cell walls of mosses often become brittle if the material is imbedded in paraffin, and cut poorly, so celloidin is often preferable. Picric acid fixing formulas are helpful here, following with hematoxylin stains. Fleshy, unchambered Hepaticae may be difficult to infiltrate in celloidin and do best in paraffin, especially for apical cells (Ricciaceae and Marchantiaceae), which are best shown in vertical median longitudinal sections. For the morphology of Riccialaceous thalli celloidin is best if cut fairly thick. Sexual organs do very well in paraffin. Sporangia are less readily cut as they become older, and should be punctured or cut open so that the reagents may penetrate. If old and horny it may not be possible to cut sections even in celloidin, and it may be necessary to treat the material like hard woody tissue (p. 162).

### XVI. Pteridophytes

For general preservation, cytological fixation, histology of the vegetative organs, etc., the general methods are quite efficient, since the types

of tissue present approximate those of phanerogams, for the preparation of which the more general sections were designed. The leaves of some Lycopodiales will give trouble in cutting if paraffin-imbedded. This extends to the comparatively young strobili, which often give trouble, and particularly in old stages where the sporangia and spore walls become hardened. The strobili should be trimmed flat from opposite sides to facilitate penetration of reagents. Axial sections are usually to be preferred. *Equisetum* stems present the difficulty of silica impregnation, and must be desilicified (p. 161) and imbedded in celloidin for really good sections. The growing apex will cut well in paraffin without special preparation. Filicales offer several points worth notice and caution. Root tip sections for apical cells are best cut absolutely median and longitudinal so that the segments may show to best advantage. For these, if cytological features are not desired, chrom-acetic fixation followed by Delafield's hematoxylin is a most efficient, simple technique. Stem tips may be similarly treated, trimming away the tough ramental scales. Older roots and stems are handled by the general methods for firm or woody tissues. Leaves may be sectioned fresh, or for more accurate details imbedded. Old sporangia with thickened annulus will give some trouble in paraffin sections, but young stages cut perfectly well. For classroom preparations median sections through the stalk of the indusium give most diagrammatic results. For cytological purposes it is well to watch that penetration be assisted in all possible ways, since the sporangial coats, especially of the Eusporangiatae, are rather resistant to fixing fluids (p. 213). Whole mounts of leaves, especially of the lobes of thin, large leaves with sporangia, are very satisfactory for showing the structure of the sorus. Clear the leaf by partial maceration in caustic potash, wash and stain in safranin. Destain cautiously and counterstain with methyl or light green; differentiate, dehydrate and clear carefully and mount in balsam. The counterstain should be rather dilute. Old indusia, annulus and mature spores will stain red, as often will epidermal glands and hairs, vascular bundles red to purple, other structures green. The flat prothallia and the protonema of ferns do well if fixed in Flemming's fluid and stained in Heidenhain's hematoxylin and orange G before mounting in balsam by infiltration, or they may be imbedded and sectioned for details of the sexual organs. The heterosporous Pteridophytes offer special difficulties in the hard walls of mature spores and spores containing gametophytes. As long as it is possible to cut at all, paraffin will probably be found the most satisfactory imbedding medium. Sections (as often in Pteridophytes) may not stick well to the slips unless a gum or gelatin affixing medium (p. 183) be employed.

## XVII. Gymnosperms

The Gymnosperms like the Pteridophytes, are in most anatomical features readily adapted to the general methods of study, so that reference should be made to the first portion of the botanical chapter. A few special topics come up to be dealt with separately. These mainly relate to the handling of the megasporangial structures, the female gametophyte and the embryo.

In the Cycadales and Ginkgoales it is necessary, if sections are to show the general topography of the ovule (megasporangium, etc.), that segments be cut from opposite sides to allow more ready penetration of the fixing fluids. These should be cut as deeply as possible, so that the cut surfaces are close to the particular structures developing at the selected stage (megaspore mother-cell, endosperm, embryo), but with a razor blade to avoid pressure on these structures, which in their early stages are readily deformed. For more accurate fixation the upper portion of the endosperm or of the nucellus containing the stages especially desired should be cut out and fixed separately. The ovule will cut well enough in the paraffin until the stony layer becomes hardened. For the free nuclear stages especial care is required to keep from causing shrinkage, and a high percentage of osmic acid will be helpful in giving a thorough preservation of the cytoplasm. The pollen tubes with the sperm mother-cells (or the sperm) in their tips are best secured in this way, or (*Ginkgo*) by cutting away the lower part of the ovule and then slipping off the upper part of the nucellus, which comes away as a cap. At the apex of this the pollen tubes are well exposed for easy penetration of the fixing fluid, which should be a Flemming type (p. 218). They may be imbedded, sectioned and stained with Heidenhain's hematoxylin, which shows the centrosomes and blepharoplasts well. Chamberlain<sup>50</sup> gives useful data on the times of maturation of the various stages. With *Pinus* it is necessary to dismember the cones for all but the very earliest stages in the first spring of their development. The individual scales soon become hard at the tips, and can advantageously be trimmed (after imbedding) back to the ovules. In many Gymnosperms the resin masses in the tissue interfere with staining procedures. They are difficult to remove from imbedded material in either xylol or alcohol, but a mixture of xylol and absolute alcohol will clear them up readily. In old stages containing embryos the ovules may be isolated immediately and even trimmed on the side to facilitate fixation. Buchholz<sup>51</sup> has given directions for the isolation of the

<sup>50</sup> Chamberlain, C. J. *Methods in Plant Histology*. Chicago, 1924, p. 269.

<sup>51</sup> Buchholz, J. T. *Bot. Gaz.*, 66: 185, 1918.

embryos of *Pinus* and other genera. Dissection from living material is best. The gametophytes are first isolated. Hold gently with light forceps by the broad end, and make a circumcissile cut about the narrow end with a needle shaped to an arrow-head tip and keenly sharpened. This end is gently removed, when by teasing into the end of the ovule the rosette ends of the suspensors are exposed and should be pushed out by the straightening suspensor shafts. By successive segmental cuts the distal portion of the suspensors, and eventually the embryos, are exposed, and finally the whole embryo complex removed. Dissection should be under a 0.3 gm. mol. sugar solution to prevent bursting of the embryo cells. Fixation in formol-alcohol is recommended, followed by staining in Delafield's hematoxylin or safranin. This is a proceeding for careful hands, the embryos being transferred with wide-tipped pipettes. If staining is accomplished in a small drop of stain, later to be flooded with water (or alcohol) before pipetting off, the embryos can be much more readily located. They are then dehydrated through glycerin and infiltrated in balsam or Venetian turpentine (p. 202).

### XVIII. Angiosperms

1. **Pollen investigations** in connection with genetical studies are often desirable to determine the percentage of normal grains developed and the types of abnormal ones. Direct microscopical examination will indicate badly shriveled grains, megacytes, etc. Germination tests will give additional information respecting pollen activity (below). Measurements of the length and growth rate of the pollen tubes in the styles (p. 244) will show the probability of the sperm being able to reach the embryo sacs. For more detailed information on grain sizes micrometer measurements may be made. For information on viability based on the stored food content the method presented by Blakeslee and Cartledge<sup>52</sup> may be tried:

"Flowers to be examined were collected in the morning, generally before the anthers had opened. The pollen was taken out of the anther with a needle and distributed in a drop of 45 per cent acetic acid slightly colored with iodine. This medium stains the contents of the grains but leaves the walls practically uncolored. Grains were recorded as abortive which were empty and shriveled. There is usually no difficulty in distinguishing bad from good grains. Counts were made of the two kinds of grains in a series of different fields of the microscope under low power. The abortive grains tend to collect at the edges of the drop. Their distribution was made more uniform by stirring with the needle and the counts were made more representative of the actual condition in the flower by examining, in succession, fields from one side of the preparation to the other."

<sup>52</sup> Blakeslee and Cartledge. *Proc. Amer. Acad. Sci.*, vol. 12: 1926.

In measurements care should be taken to make the observations soon after putting the pollen in the acetic solution, for even with the most cautious adjustment of the strength of the acid, swelling even to bursting, or shrinking, will result after thirty to sixty minutes. After familiarity with the material has been secured, differences in chromosomal number may be detectable in differences in the size of the pollen grains (courtesy of Cartledge). Chamberlain recommends for loose pollen of anemophilous plants that it be soaked for fifteen to twenty minutes before fixation in order that it may become turgid before fixing. Afterwards it may be fixed in bulk like minute organisms, washed by sedimentation, stained, dehydrated and infiltrated with balsam, or after dehydration imbedded and sectioned.

**2. Pollen tubes** are generally studied by culture methods.

Most pollens quickly lose their vitality, so germination should not be unnecessarily delayed. Some kinds grow well if simply spread on parchment paper or clean glass slips and maintained in a water-saturated damp chamber. Others require water, the drop being allowed to spread thin on a chemically clean slip so that oxygen may be more readily available. For most species success attends culture in a solution of cane sugar, the optimum strength of which varies with the plant over a wide range: *Papaver*, 1 per cent; Compositae 30 to 40 per cent. The sugar may be mixed with 1 to 2 per cent agar and the mixture allowed to stiffen on slips or in Petri dishes. After inoculation the slides must be kept in a damp chamber and the Petri dishes closely covered. The agar may be cut up into blocks and these sectioned after imbedding.

Pollen tubes in the pistil of a pollinated plant are best studied in position. The pistil may be cut off and fixed, and the tubes located in longitudinal paraffin or celloidin sections. The usual fixing fluids and imbedding methods may be adapted, but because of the general presence of air and spongy tissue in the style it is advisable to split it before fixing and to remove the air with an air pump. The common cytological staining methods may be employed for studies calling for such details, but for histological purposes a combination involving aniline blue is recommended, since this stains the callose of the tube wall especially readily from the dilute solution. A particularly valuable method has been perfected by Buchholz and Blakeslee<sup>53</sup> particularly for *Datura*, but widely adaptable. Their method may be quoted as follows (courtesy of J. L. Cartledge):

"The styles were . . . scalded in hot but not boiling water (about two minutes), their cortexes slit lengthwise by passing them through a groove in which the sharp corner of a razor blade protruded slightly. This treat-

<sup>53</sup> Blakeslee, A. F. *Science*, vol. 55: 1922.

ment facilitated the removal of the cortical tissue by dissection, leaving only the central strand of conducting tissue with which the stigma is continuous at the end. These central cores were stained in Magenta (Acid Red), washed a little in water, and mounted whole on a slide, using concentrated lactic acid as a mounting medium and clearing agent. Balsam mounts were found not satisfactory but these lactic acid preparations have kept for more than six months. Pressure applied to the cover glass will spread this tissue out in a thin layer, and the pollen tubes may be seen even under low power (better after twelve to twenty-four hours) as dark red streaks embedded among the elongated pink stained cells of the conducting tissue. Germinated pollen grains are transparent and may be recognized only by their empty shells (the extine walls) while the ungerminated pollen will stain a deep red. This method makes possible reliable counts of the number of ungerminated pollen grains and the number of pollen tubes at any given time after pollination."

The use of lactic-glycerin (p. 198) instead of the simple lactic acid may be of some advantage as it is of slightly higher refractive index. The mounts may be sealed with the usual cements (p. 199).

Nebel has described methods of using lacmoid and martius yellow<sup>54</sup> for staining pollen tubes in flower styles which seem to offer promise of usefulness in cytogenetic problems. A stock solution of 5 mg. lacmoid and 5 mg. martius yellow are made in 10-15 c.c. of water, which solution is brought to a deep olive green color by a few drops of 1 per cent ammonia solution. After two to five minutes of staining the styles are mounted in the dye or in water of the same pH. For permanent mounts styles are crushed, dissected or otherwise made ready and fixed in Carnoy's solution (p. 217). They are then passed through 70 per cent alcohol to alkaline water, rinsed in tap water and stained in the above mixture for one to five minutes. Dehydration should be rapid and the alcohol should contain lacmoid at pH 8, when the solution is blue. Clearing should be done through xylol and mounting should be in balsam.

<sup>54</sup> Nebel, B. R. *Stain Technology*, 6: 27, 1931.

## CHAPTER V

### CYTOLOGICAL METHODS

C. E. McCLUNG, E. ALLEN, R. T. HANCE, J. W. McNABB, E. V. COWDRY

GENERAL CONSIDERATIONS 246. GENERAL METHODS 248. Fixation 248. Washing 253. Dehydrating 253. Clearing 256. Infiltrating 257. Sectioning 258. Spreading 259. Staining 260. Collodion sections 261. Smear methods 262. Aceto-carmin method 264. SPECIAL METHODS 265. Mitochondria 265. Golgi apparatus 274.

#### A. GENERAL CONSIDERATIONS

The development of cytological studies has reached such a stage that it has now become a matter of very great importance not only to have methods of the highest character, but also to make such a selection of material as will profitably repay study. Some consideration of the character of the material may therefore be worth while.

1. **Selection of material** should be discriminating. Sufficient studies have been made of the different plant and animal groups to indicate their cytological characteristics in a general way. Before undertaking an investigation, therefore, a review of the literature dealing with the different groups should be made in order to discover the best kind of material available for any particular purpose. While it is desirable to investigate any new types that present themselves, if a particular subject requires consideration it is wiser to select a form which has already demonstrated its worthiness. There are many elements that enter into such a decision, among which are the availability of the material at different seasons of the year, and convenience of handling in the laboratory. Sometimes, however, a form is exceedingly valuable, for instance, as genetical material and it then becomes necessary to discover, so far as possible, its cytological characteristics. Under such circumstances the best has to be made of poor material oftentimes, and the only recourse here then is to make the best possible use of our technical methods. When, however, a choice can be made, as has been indicated, the element of selection enters largely into the final results.

2. **Preliminary treatment of specimens** has much to do with the end result. If the material has not been fixed directly after being brought in from the field, specimens should be carefully housed in approximately normal living conditions. If we are dealing with animals, they should be kept in cages which are appropriate to their size and habits. In many cases it is of great importance how they are handled. For instance, it has

been found in the case of the albino rat that if animals are gentled they exhibit very different reactions from those which have not been subjected to this preliminary training. While these differences are apparently largely physiological, they must have a structural basis. Physical conditions, such as food, light and temperature, also have marked influence upon the morphological characteristics of animals. Sometimes these influences are of a very profound sort. In the case of certain grasshoppers which were raised in the laboratory, if the animals were fed exclusively upon clover the cytoplasm of the germ cells became loaded with dense materials which were absent when the animals were fed upon a diet to which they were normally accustomed. In the absence of proper cleansing and light conditions, fungi may attack the animals, and, for the grasshoppers mentioned, it had much the same effect as followed feeding with clover.

3. **The condition of specimens at the time of fixation** requires consideration. The age of the specimen, of course, is directly related to the state of development of its cells, and an appropriate selection should be made in order to get the stages required. Only a knowledge of the rate of development will make this exactly possible, but in most cases, if studies of the germ cells are involved, an understanding of the breeding habits of an animal will indicate roughly the time at which the material should be put up. Even within the same group, however, there are marked differences in cellular history so that, for each species, it is necessary to acquire some familiarity with the type. Amongst the grasshoppers, for instance, there are certain ones, like the *Truxalinae*, where the adult animals show a quite satisfactory succession of developmental stages, whereas in the *Acridinae* the cells pass more rapidly through their development and show only later stages in the adult condition. In this latter case the cells give indications of senility and the conditions are not normal. Due care should therefore be exercised to see that the animals are in the proper stage to present cells of normal constitution. In the Hymenoptera, unlike many other insects, it often happens that all the cells of the gonad are in the same stage of development. Hance reports that by shaving off the hair of mice many dividing cells may later be found by sectioning the follicles.

Another matter which has to be considered is the metabolic condition of the specimen. It sometimes makes a great deal of difference whether the animal has been fed or starved. In general, of course, it should be in the healthy state which follows normal feeding, but in some studies conditions are revealed after starvation that are important for an understanding of normal cell functions. A feature of very great importance, which is often overlooked, is the length of the period between death of



the animal and the preservation of its cells. As a rule, the material should be brought as rapidly as possible into the fixative, and it is often advantageous to apply the reagent to the cells *in situ*. As was indicated in Part I the method of killing also has an important bearing upon the structural conditions of the cells.

## B. GENERAL METHODS

### I. Fixation

A general consideration of this subject will be found in Part I and also in the chapters on Fixatives and Fixation, and Botanical Methods. In cytological work, however, the choice and use of the fixative is of such significance that some special consideration may be warranted. In the first place it is necessary to decide whether the exact presentation of nuclear structures or of the cytosomic constituents is more important. While it is not true that there is such a marked difference in the particular reagents required as is commonly believed, yet there are certain practical considerations which operate in the choice. If one is inexperienced in the characteristics of the particular material which it is intended to study, the wisest procedure will be to select first one of the *picro-formol-acetic* combinations. The advantages of these, as is elsewhere indicated, are that they do not overfix and they are perhaps more generally adaptable than any other class of such fixatives. Their main disadvantages are that they do not preserve well all of the cytosomic elements, and besides certain nuclear aniline stains do not take so vigorously as after treatment with osmic acid mixtures. For these reasons it will generally be found advantageous to supplement the *picro-formol-acetic* fixations by others with *chrom-osmic-acetic* mixtures in which there are present varied proportions of acetic acid. These two classes of fixatives will be satisfactory in a very large proportion of the cases, even for final studies. After experience has been gained with a particular type of material it will be profitable many times for the investigator to experiment by varying the proportions of the ingredients which enter into the combinations. In extended studies of Orthopteran material and certain plants it has been found that these proportions have a marked influence upon the relative density of cell parts as well as upon their finer details. Also, as will appear in the fuller discussion under Fixatives and Fixation, and Botanical Methods, the presence of certain adjuvants like urea and sugars have pronounced effects when added to the fixatives. Since the value of all later steps depends upon the quality of the fixation, it becomes of first importance for the investigator to exhaust all of the possibilities of im-

provement at this stage before finally settling down to a study of the material. It cannot be too strongly emphasized that exact fixation is a primary requisite in all cytological work.

1. **Special Methods.** Special techniques have been developed in extensive studies of certain animal groups, some of which present unusual difficulties. Much of the work done upon mammalian cytology is valueless because the process of fixation has been improperly conducted. Within recent years two general methods of treating mammalian cells have been developed which produce excellent results. Already these have been given such thorough trial, under a great variety of conditions, that they may be said to have established themselves as the principal reliance in all studies where great exactness in the preservation of fine cytological details is required. These techniques involve not only a consideration of particular reagents, but also certain niceties in their use.

Flemming's fluid and Allen's B-15 have proved the most satisfactory fixing fluids for mammalian tissues, especially for chromosomes. In the United States the use of the former at near the freezing point has been found helpful. This method is described by Hance (p. 250). Allen's B-15 has been used by many workers and found very satisfactory.

a. *Allen's Special Methods. Time of fixation in B-15* (for formula see p. 561) is usually one hour, if outer membranes have been removed and the fluid held at 38°C. This is based upon pieces of such tissue as rat testis and young rat brain not over 0.5 c.c. in volume. Longer fixation seems unnecessary and may be detrimental. After this time, some users have found that placing the tissue in Bouin's fluid is helpful. While the penetration of B-15 is better than that of Flemming's fluid, it is best to apply it to small pieces of tissue. Snip rat testis with the scissors so that many surfaces are exposed. Brains of very young rats do not much exceed 0.5 c.c. and they may be cut in half by sagittal division at time of fixation by using a thin safety razor blade or they may be otherwise divided in order to let the fixative attack an unprotected surface.

If the tissue is held a few days in 70 per cent alcohol, with frequent changes, it is not only hardened but is freed from the remaining picric acid. The use of a few drops of a saturated solution of lithium carbonate at intervals hastens the removal of the picric acid. While some workers find the presence of picric acid not detrimental, my experience with mammalian tissue is that if much is present, infiltration with paraffin may be difficult and that thick sections ( $20\mu$ ) do not adhere well to the glass. The higher grades of alcohol may be replaced by aniline with less shrinkage, as described on p. 256.

*Other fixatives.* B-20. I have recently found that a little 2 per cent osmic acid added to the B-15 mixture improves its action on mammalian

chromosomes. As commonly made up, only half the chromic acid called for by the formula (p. 561) is used, and to each 50 c.c. is added 1 c.c. of Flemming's solution B. (1 gm. osmic acid in 50 c.c. of 1 per cent chromic acid. This is the equivalent of 2 per cent osmic acid.) For convenience it is named B-20. The after treatment is the same as for B-15. This fluid has been found very satisfactory also for eggs and spermatogenic tissue of the Cladoceran *Moina macrocopa*. Mix at time of using.

*Double fixation*, or the use of two fixing fluids, has been found useful in some cases. If much fat is present, Carnoy's fluid is first applied for from ten seconds to several minutes. This is followed by any other fixative. In *Moina macrocopa* eggs I have found that this treatment followed by B-15 eliminates the oil globules and fixes the remaining yolk substance and chromosomes very well.

*General Remarks. Duration of Fixation.* This varies greatly with different fixatives and tissues. Specimens remain indefinitely without injury in Bouin's fluid, but harden too much for good sectioning if left in B-15 over two hours. Most people fix twenty-four hours in Flemming's fluid, but Professor H. de Winiwarter (Liège, Belgium) fixes from forty-eight to seventy-two hours. His formula is somewhat different from that in general use in this country. It follows:

(a) Osmic acid .....	4.0 gm.
Chromic acid .....	7.5 gm.
Distilled water .....	950.0 c.c.

(b) At the time of using add 3 or 4 drops of glacial acetic acid to 20 c.c. of (a), or, as seems better, 6 or 8 drops of trichloroacetic acid. He washes for twelve to twenty-four hours, and dehydrates carefully by graded steps, and clears in cedar wood oil (H. von Winiwarter, 1912). I have seen his preparations of human testis by this method and the chromosomes are beautifully distinct in all stages (Ezra Allen).

For a method of injection and methods of subsequent treatment see page 566.

*b. Hance's Method for Mammalian and Avian Material.* Flemming's strong solution to which has been added about one-half of one per cent urea, when chilled to the temperature of ice, gives an excellent preservation of the chromosomes in avian and mammalian cells. This mixture does not penetrate well, which necessitates the use of pieces of tissue preferably not larger than a small pea. In the case of chicken tumors, which are extremely dense, slices as thin as can be cut free-hand with a razor are dropped into the fixative. Chicken testes, which are also difficult to penetrate, should be thoroughly teased after being placed in the fluid. Avian and mammalian embryos have all their structures well preserved by this method without the necessity of teasing to aid penetration. Ap-

appropriate pieces of tissue are placed in the fixative. (For formula see p. 559.)

Mix as needed, so that for every piece of tissue the size of a small pea from 10 to 15 c.c. of fluid is available. Place the bottles containing the fluid in cracked ice.

Fix four to twelve hours.

Wash in running water for twelve hours.

Dehydrate by the "drop method," displacing the water drop by drop with alcohol. While 70 per cent alcohol may be used for this purpose and the tissue stored in it I consider it more convenient to use 95 per cent alcohol and imbed the material at once. When the tissue has reached 95 per cent alcohol, change to fresh 95 per cent at least once, twice being better. Tissue remains in the last change of alcohol for from one to two hours.

*Clearing.* Cedar oil or oil of bergamot is used for clearing. It is preferable to add it to the alcohol drop by drop although satisfactory results are usually obtained by adding relatively large fractions to the alcohol which covers the tissue. Change the tissues to fresh oil two or three times. They may stay indefinitely in cedar oil although over night is usually a convenient interval for either oil. Before infiltrating with paraffin the oils may be either washed out with chloroform or the tissue placed in the paraffin directly. When chloroform is used it is allowed to act from thirty to sixty minutes.

*Imbedding Medium.* Paraffin. The chloroform-covered tissues are warmed and melted paraffin is added several times during a two to four hour period. The tissues are transferred to pure paraffin and allowed to remain for from two to four hours. They may be handled in the same way when chloroform has been omitted, although the time allowed for infiltration must be longer as it takes more time to displace the heavier oils.

*Section Thickness.* Avian tissue gives best results when cut at  $5\mu$  and mammalian when cut at  $10\mu$ .

*Staining.* The slides are bleached of the osmic acid stain in 70 per cent alcohol plus peroxide in proportion of about 4 parts of alcohol to 1 part commercial hydrogen peroxide. The slides remain in this solution twelve hours or longer. Iron alum hematoxylin has given the sharpest results for cytological studies (R. T. Hance).

*c. Carothers' Method for Orthopteran Cells.*

Picric acid, saturated aqueous solution .....	75 c.c.
Formalin, C. P. ....	15 c.c.
Glacial acetic acid .....	10 c.c.
Urea crystals .....	1 gm.

Warm slightly and stir thoroughly as the urea is added.

This solution is used if the material is to be kept in the fixative. It is satisfactory for work involving metaphase conditions.

For preservation of the finest cytological details add 4 drops of a 50 per cent aqueous solution of chromic acid to 5 c.c. of the above solution just before using. The tissue must then be removed from the fixative after ten to twenty-four hours, washed thoroughly in tap water (one or two hours) and run up to 70 per cent alcohol where it may be stored after most of the picric acid has been removed.

In case one wishes to use Flemming's triple stain, osmic acid must be added. Five drops of a 2 per cent solution to 5 c.c. of fixative is sufficient.

**2. Methods of Application.** It is sometimes a matter of great importance how the fixative is applied to the tissues. As is elsewhere indicated, there are two general methods, that of immersing the material directly in the fixative and that of injecting the fixative into the organ or whole animal to be studied. Sometimes in the case of smaller animals the whole organism is immersed in the reagent. It is not unimportant how the fixative is applied by immersion. For instance, in studies on *Culex*, some investigators reported the diploid number of chromosomes as 3 and others as 6. Upon careful investigation of the cause of these differences it was found that if the fat and trachea were not removed from the testis, the fixation was imperfect and the chromosome pairs ran together, reducing the number from 6 to 3. Although the organ here is very small, and one would naturally think that fixation might be easily accomplished, the neglect to remove the fat and trachea was responsible for such an important difference as this. It should be the rule, therefore, so to manage the process that the fixative is brought into as direct and immediate contact with the tissue as is possible. A further discussion of this topic will be found in the chapter on Fixatives and Fixation.

In the case of large organs it is important to effect this intimate contact between the reagent and the tissue by injection through the circulatory apparatus. The difference in the effect produced by fixation of mammalian testes through immersion and by injection is very marked. Ordinarily, in the former case, the histological details are very poorly preserved, there being extensive shrinkage of the germinal elements from the connective tissue about them. In a preparation well preserved by injection, on the contrary, the shrinkage is entirely absent and the cells are everywhere in intimate contact. In studies upon large organs, therefore, it should always be the endeavor to try out the process of fixation by injection. It is described in detail by Allen in the chapter on Fixatives and Fixation.

3. **Physical Conditions during Fixation.** The concentration of the fixative, the proportion of its parts, the presence of adjuvants and the degree of temperature, are all elements that require extreme care in cytological technique. These are discussed at length in Part I and in the chapters on Fixatives and Fixation, and Botanical Methods. It is perhaps sufficient to call attention to these points here and to emphasize their great importance.

4. **Period of Fixation.** This is determined by the size of the specimen and by its character. In general it is desirable not to prolong the action beyond the time necessary to secure accurate fixation. This varies also with regard to the character of the reagent. It is quite impossible to give any general rule which can safely be applied to all types of material and to the reaction of all fixatives, but in general it may be said that a piece of ordinary tissue not exceeding 5 mm. in diameter may be fixed within two to twelve hours with safety in most fixatives. In special cases, on the contrary, it is often desirable to prolong the period of fixation to great lengths in order to secure specific effects. This is particularly true in the case of mitochondrial studies and in certain neurological practices. It is perhaps a safe general plan to start with a minimum period and extend this only as experience indicates that it is desirable.

## II. Washing

A description of this process will be found in Part I and also in the chapter on Fixatives and Fixation. It will suffice here to indicate that in cellular studies extreme care is necessary, since, after chrom-osmic-acetic fixations, for instance, either a mitochondrial stain or a chromatic stain may be secured with hematoxylin, depending upon the extent of washing. Puzzling artifacts may result from insufficient washing, especially after the use of mercuric chloride. In general, the quality of staining is much influenced by the degree of washing of the fixative.

## III. Dehydrating

Even the best fixation may be spoiled by after treatment and this is particularly true during dehydration. The absolute rule here is that the transfer of the material from aqueous solutions to alcoholic must be made in the most gradual manner. To accomplish this the tissues may be run up through a finely graded series of alcohols allowing sufficient time in each for a complete replacement of the lower percentage of alcohol, or, preferably, the alcohol may be added drop by drop to the water in which

the specimen is placed until the concentration of alcohol is high. A description of this method is given by Allen as follows:

The following method has proved very satisfactory for maintaining

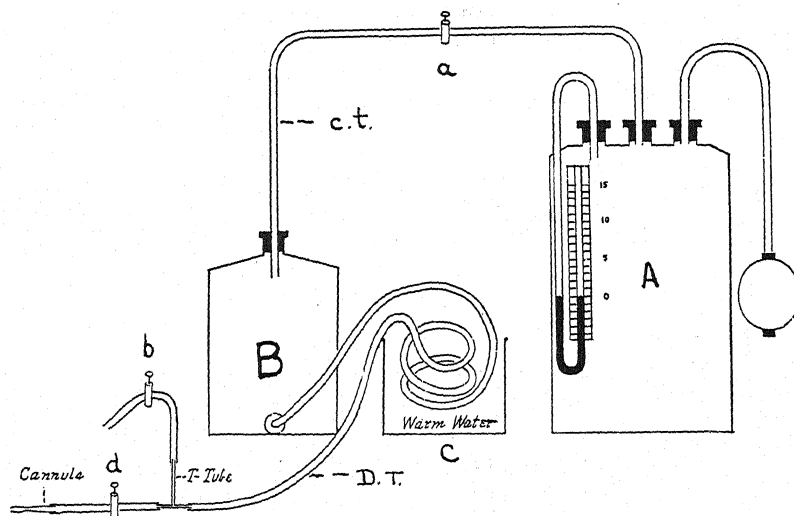


FIG. 1. Apparatus for injecting.

the normal relationships between tissues of considerable difference in texture, as the interstitial cells and the tubules in rat testis, and mesenchyme and ectoderm in mammalian embryos. (Allen, 1919.)

The new fluid is added one drop at a time, while the fluid containing the tissue is agitated by either mechanical stirring or by a current of air bubbling through it. The agitation insures that the surface of the tissue is constantly bathed in a fresh fluid. Bubbling air may be introduced from any source of compressed air. The accompanying cut (Fig. 2) shows an adaptation of the pressure bottle for this purpose (P.B.). One may substitute an atomizer bulb connected to the pressure bottle for the water bottle (W.B.) as shown in Figure 1. Sufficient pressure to last for some time may thus be obtained. A slight current in the fluid is sufficient to distribute rapidly the new drop of fluid as it enters the container (C). An automatic siphon (S) carries away the excess into a waste jar (Waste). The sulphuric acid drying bottle may be connected in the air tube when adding the clearing agent.

The following brief outline of procedure is based upon the treatment of a piece of tissue about 0.5 c.c. in volume which has been fixed in B-15. Agitation is understood to be maintained throughout the process, even if no fluid is being added. The new fluid is added at the rate of one drop

per second unless otherwise stated. The left hand column indicates the fluid in which the tissue is at that stage. Remove the tissue from the fixing fluid, rinse in 5 per cent alcohol, and place in 5 per cent alcohol.

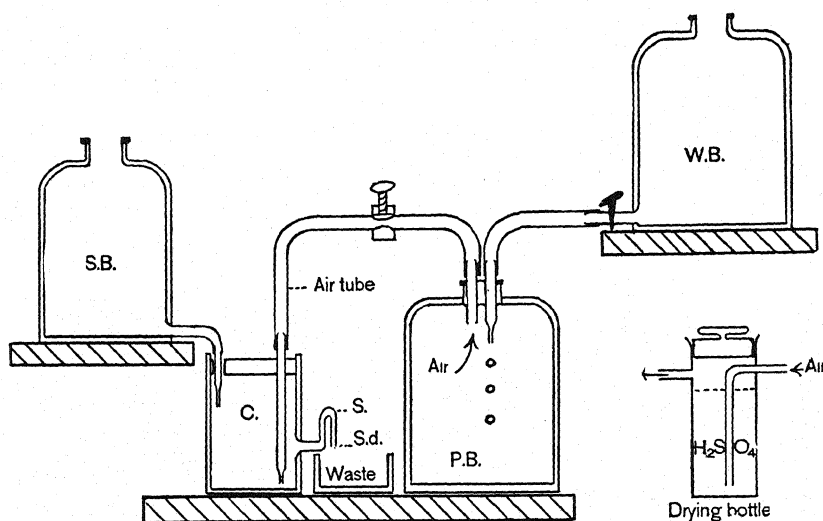


FIG. 2. Agitator.

1. 5 per cent alcohol. Drop in 10 per cent alcohol plus a few drops of saturated solution of lithium carbonate, until tissue is in practically 10 per cent alcohol.

2. 10 per cent alcohol plus lithium carbonate. Let remain two hours with agitation.

3. Fresh 10 per cent alcohol. Drop in 50 per cent alcohol plus about 1 per cent lithium carbonate until tissue is in approximately 30 per cent alcohol.

4. 30 per cent alcohol. Drop in 50 per cent alcohol plus an equal part of aniline, one drop about every five seconds, until the tissue is in approximately the new mixture.

5. 50 per cent alcohol and aniline, equal parts. Drop in equal parts of 70 per cent alcohol and aniline, one drop in five seconds.

6. 70 per cent alcohol and aniline, equal parts. Drop in pure aniline still more slowly—one drop in ten seconds. Continue until tissue is clear throughout, when dehydration is complete, and clearing may be begun by the same method.

If a whitish precipitate appears at any step after aniline is begun, it indicates that not all of the fixative has been removed, as aniline and the fixative are incompatible. In such cases the tissue must be returned to the former fluid for a longer time. If alcohol instead of aniline is employed, as it may be for some relatively hard tissues, removal of all the fixative is not necessary. (See remark on this subject on page 249.) Do not use aniline after Flemming's fluid. The tissue turns very dark and seems impossible to bleach.



Dehydration may be carried on by the more laborious method of adding the new fluids by 5 per cent or in some cases 10 per cent steps without agitation, but it is time-consuming.

If large numbers of pieces of tissue are to be done at once, the apparatus described on p. 571 may be used.

#### IV. Clearing

The process is an intermediate step between dehydration and infiltration in the paraffin method, and grades by varying degrees, according to the reagents used, into these other steps.

Dehydration is commonly completed in alcohol, but in the case of delicate tissues for cytological work it is best to avoid the higher grades of alcohol and to utilize some such agent as aniline oil or dioxan in order to escape their stronger action. The processes, therefore, cannot be regarded alone, but must be considered in relation to dehydration and infiltration. If dehydration has been completed in 95 per cent alcohol or absolute, there are available quite a number of clearing agents which may be utilized to displace the alcohol. For many cytological purposes the benzene ring series, benzol, toluol, and xylol are particularly appropriate preceding the paraffin bath. For mammalian testes Allen has found that methyl-salicylate gives unusually good results, while for brain tissues he prefers bergamot oil.

Following fixation in B-15 his experience has been that aniline is most desirable and refers to the method as follows:

"For most purposes alcohol is an adequate dehydrating agent. However, for mammalian chromosomes fixed in B-15 the use of aniline has proved very helpful (Allen, 1916; Painter, 1924), as it produces less shrinkage. The purest aniline obtainable is usually somewhat reddish, but by distillation it becomes nearly colorless, and does not stain the tissues. Aniline is perfectly miscible in 50 per cent alcohol equal parts. This mixture passes readily into 30 per cent alcohol. Hence pure alcohol higher than 30 per cent may be avoided." (For details of method see p. 253.)

If aniline is used to complete dehydration and for clearing it is best to remove this with some agent like chloroform of higher volatility and greater miscibility with the paraffin.

For very delicate cytological work the same precautions that apply to dehydration also hold for clearing. It is necessary to avoid sudden changes in concentration of fluids and to minimize distorting effects of diffusion currents set up between different substances. Accordingly there must be provided a gradual passage through a series of mixtures of different relative concentrations, or the addition of the clearing fluid

drop by drop to the dehydrating agent in which the specimen is placed. Allen's description of the drop method of dehydration therefore can be applied to clearing.

Whatever the agent employed or the method of its application, care should be exercised not to expose the tissue longer than necessary to the action of the clearing medium. Practically all such substances have a shrinking and hardening effect which should be minimized by the briefest possible action. If the essential oils or aniline are used the completion of the process can be determined by the translucent appearance of the mass of tissue (see Part 1). Careful experimentation will reveal for any particular tissue the clearing medium which will produce the least shrinkage and in this manner it can almost be eliminated. Thus cinnamic aldehyde will clear delicate chick embryos with practically no reduction in size whereas xylol will reduce them to three-fourths their original bulk. When delicate cellular detail is involved no pains should be spared to preserve this against distortion by all reagents employed.

#### V. Infiltrating

It is a general practice amongst botanical cytologists to make a very gradual substitution of the clearing agent by paraffin. (See the chapter on Botanical Methods.) Such a procedure is less common amongst animal cytologists, but in many cases it is doubtless true that a better result would be obtained than by the method of transferring the material directly from the clearing fluid into the melted paraffin. In case of many animal tissues, however, it is quite satisfactory to make such a sudden transfer and thereby the hardening action of the clearing agent is somewhat minimized. When new material is being investigated it would be a good practice to try out the two methods and follow the one which gives the better results. The danger involved in the use of paraffin results from its hardening action when melted. Some workers, therefore, prefer to infiltrate first with a paraffin of low melting point, following this by final immersion in the higher grade desired for sectioning. The endeavor should always be to reduce the exposure of the tissue to the melted paraffin to the lowest limits and also to use the softest paraffin that will produce good sections. The latter alternative, however, has very limited range. Since higher temperatures produce the most disturbance in the tissue, the temperature should be kept as near that of the melting point of the paraffin as possible. There are numerous mechanical devices for securing this condition, but the simple overhead application of heat described in Part 1 gives a most valuable and flexible means for securing this result.

What has just been said with regard to the harmful effects of melted paraffin at high temperatures is undoubtedly true in most cases, but Foot and Strobell<sup>1</sup> report that the eggs of *Allolobophora* are not injured by the use of paraffin melting at 74°C. Quite in contrast to usual practice also, in order to get very thin sections, they cut at a temperature of 20°F. Like all other rules on technical matters the ones relating to handling paraffin must be general, and entirely subject to modification according to experience.

In his work on mammalian cells Allen finds that the process of infiltration by degrees is as important as any other step. It should be at as low a melting point as possible for sectioning. A combination of ordinary parowax with about 4 per cent bayberry wax is recommended as an excellent medium. Pure parowax is used by many workers instead of that prepared for a certain melting point. Rubber paraffin (p. 621) with a low melting point is probably the best, as it is very flexible at any temperature or thickness of ribbon. Small blocks may be cooled on ice and sectioned at 5 $\mu$  on a warm summer day, when ordinary paraffin would be too soft. If possible, keep below a temperature of 52°C.

The paraffin may be first added to the clearing agent in small pieces, and these allowed to dissolve at room temperature. Chloroform has the advantage of absorbing a large amount of paraffin at this temperature. Some workers saturate it before applying heat. Keep tissues in the melted paraffin only long enough to insure complete infiltration. Shrinkage is likely to be great in any case.

If collodion is used instead of paraffin, less shrinkage results than with paraffin at its best. Follow either 100 per cent alcohol or aniline with a mixture of equal parts of 100 per cent alcohol and ether, and this fluid with 2 per cent collodion. Use plenty of this and let it slowly evaporate to a stiffness suitable for sectioning. The evaporation should take from ten days to two or more weeks.

## VI. Sectioning

Sections which are quite satisfactory for ordinary histological work may be entirely unsuited to use for finer cytological studies. Unless the conditions are of the very best with regard to the character of the material, the relation between the material and the imbedding medium and the perfection of the knife edge, the sections will be faulty. Especially in chromosome studies, on material where the formed elements may readily be dislodged by an imperfect knife edge, it is necessary to use every precaution to avoid injury to the cells. These conditions become

<sup>1</sup> Foot, K., and Strobell, E. C. *Biol. Bull.*, 9: 5, 1905.

especially difficult of fulfillment when extremely thin sections are desired. Aside from having the microtome in perfect mechanical condition, and the knife edge as keen and smooth as possible, the best aid in getting exact sections is to have the melting point of the paraffin accurately adapted to the temperature of the room when the sections are cut. Finally, the inclination of the knife to the plane of sectioning should be very carefully adjusted to reduce the amount of compression to the minimum. In case very thin sections are desired the water method of Huber is extremely useful. It has been used by Heuser and is described by him elsewhere (pp. 192, 478).

The thickness of sections is dictated largely by the size of the cells present and by the character of the study to be made. Precision of staining is reduced if sections are thicker than the diameter of nuclei since differentiation is hindered by their uncut membranes. Some sections should therefore be cut thin so as to expose nuclei to the direct action of stains. If very exact studies of delicate details are contemplated, very thin sections ( $1\mu$  to  $5\mu$ ) should be made. On the other hand, if general relations are to be studied in the cell, or if counts of entire chromosome complexes are to be made, thick sections are preferable. It is a safe rule, especially in the use of unfamiliar material, to cut thin, medium and thick sections from each specimen. An inspection of readily prepared smears will indicate approximately the required thickness of sections.

## VII. Spreading

Much of the earlier cytological work was accomplished on sections which were affixed to the glass slip by pressing them down upon a thin film of Mayer's albumen. Since considerable compression occurs at the time of sectioning, especially with thin sections, much distortion of cell elements was produced. In all exact studies where the relations of cell parts are important, it is necessary to extend the sections to the original dimension of the block from which they were cut. To do this the common practice is to apply heat to the sections while they are floated upon either distilled water or a very dilute solution of albumen. By this means all irregularities are removed and the original proportions of the cell parts are restored. If the objects studied are eggs the sections should show them with circular outlines rather than oval ones. Such a test can only occasionally be applied, of course, but some means for determining the complete extension of a section is desirable. This is fairly approximated when the individual sections show the same dimensions as the surface of the block from which they were cut. This precaution is especially necessary if comparative measurements of cell elements are contemplated, for

not only is a differential compression avoided, but also the errors that are introduced by foreshortening of structures lying in folds of sections. Spreading is an operation of seeming unimportance, but upon the care with which it is carried out depends much of the value of the preparation. Here, as in all other steps of preparation, only the best attainable results should be tolerated. A good slide may serve many unforeseen purposes, whereas one which may be technically sufficient for an immediate use may be so imperfect as to be otherwise valueless.

### VIII. Staining

There are certain stains which are of particular importance in cytological work and constitute a standard resource in such studies. In the first rank of these stand the iron hematoxylin method of Heidenhain and the tricolor method of Flemming. So far as nuclear elements are concerned these two combinations suffice for almost all types of cytological work. Since a description of stains and staining will be taken up in a separate chapter, no further reference to the operation of this part of the process will be given here. (See Stains and Staining.) In most material it will be found that nuclear structures particularly, in various stages of development, stain with different intensities so that in one slide it is difficult to find optimum conditions for all. Because of this and for other reasons it is of advantage to have slides some of which are lightly stained, while others are darker. *A fortiori* the differences apparent in hematoxylin and tricolor stains of the same material make the practice of using both desirable as a matter of routine. Experience has shown that the Feulgen reaction is so important that its use should be a routine procedure. The transparency of aniline preparations and the contrasts in color they show in the same elements at different stages make them very valuable. With the improvements in the optical performance of modern microscopes the use of artificial light has become almost universal with the high powers used in cytological work. Colors which contrast well with daylight illumination often fail entirely with artificial light, so that it becomes necessary to test out the dyes which enter into combinations under the optical conditions used. For instance, the safranin formerly recommended in the tricolor stain is much too purple to contrast well with the gentian violet, and some more yellow variety is therefore required. By the choice of appropriate light filters somewhat the same effect may be secured if necessary.

Special stains are required for cytosomic constituents and these are described in the section of this chapter devoted to such structures.

### IX. Collodion Sections

In cytological work the paraffin method is used almost exclusively. This practice is largely justified by its convenience and general accuracy, but there are certain advantages in the use of collodion which would practically require that at least some of the material used in each investigation should be prepared according to this method. While it is difficult to cut thin sections in collodion, by proper manipulation this may be readily accomplished, and, by using the appropriate means, serial sections can be readily mounted. Some of the advantages in the use of the collodion method appear prominently when studies of prophase figures in the germ cells are involved. There is less danger of shrinkage than with paraffin and minute separations of parts often appear with greater precision and permanence than they do in paraffin sections. It is true, also, that after some fixatives which give very poor results when sectioned by the paraffin method, collodion sections may present accurate and brilliant pictures of normal conditions. For instance the chromosomes of Orthopteran spermatocytes after fixation in Helly's fluid showed extensive shrinkage and it was quite impossible to study them, whereas in collodion sections they were of very excellent quality.

While the paraffin method may be applied almost universally there are certain resistant objects which have so far defied sectioning in paraffin. Among these are Orthopteran eggs. For such objects the collodion method offers a welcome alternative, and, as thus applied, is described as follows by Josephine W. McNabb:

**The preparation of the freshly laid grasshopper egg** is difficult, due to the large amount of yolk and thick chitinous chorion present. Carnoy-Lebrun penetrates the chorion and yolk, giving a thorough and good chromosome fixation. The separated eggs are treated about ten minutes, allowing one cubic centimeter of fixing fluid per egg. After about five minutes the chorion is punctured with a fine steel needle. (The puncture should be made in a lateral position, thus avoiding the critical region about the caudal end. The yolk should be sufficiently hardened so that it does not exude from the puncture.) From the fixing fluid they are transferred immediately to weakly iodized 70 per cent alcohol for about twenty-four hours, or until the solution is no longer decolorized. The eggs are then removed to 70 per cent alcohol where they may be left indefinitely.

The collodion method is the only thoroughly successful one of sectioning. Paraffin is not satisfactory on account of the difficulty in cutting the brittle yolk, and because heat causes clumping of the chromosomes and

shrinkage of the large cells. The ordinary means of dehydrating and of infiltrating with collodion may be followed. It is necessary to use thick collodion and allow the infiltration to continue for two to four weeks. The chorion should be punctured in a lateral region under the collodion.

Longitudinal or oblique sections are more conveniently prepared than cross sections. In making serial sections the eggs are cut at 12 micra, preferably with a Minot precision microtome. As the sections are cut each one is pulled to the back of the knife with a sable brush. Here they are arranged serially in parallel rows. It is necessary to keep the sections and the blocked egg constantly wet with 60 per cent alcohol. When the entire egg is cut the sections are transferred in order to a chemically clean slip. This is accomplished by first pulling them onto a safety razor blade with a dissecting needle, and then onto the slip. The length of the rows should be made to correspond to the length of the coverslip used, and the same number of sections should be in each row.

After each slip is filled (an egg requires approximately two slips) the moist sections are blotted with filter paper to press out all excess alcohol. A thin solution of collodion is quickly flooded over the sections with a pipette, and this coating is immediately rinsed with a mixture of equal parts absolute alcohol and ether to remove as much of the collodion as possible, but still leave sufficient to prevent the sections from becoming displaced. The slip on which the sections are mounted is then placed in 70 per cent alcohol until it is convenient to stain. The sectioned egg, thus mounted, is mordanted in 4 per cent iron-alum, stained in a  $\frac{1}{2}$  per cent solution of Heidenhain's hematoxylin and mounted in damar. This method may be applied to other large insect eggs as well and the transfer of sections in serial order by the use of tissue paper (Part I, p. 30) may be substituted for the plan given.

The method of preparing collodion sections as ordinarily practiced is described in Part I and in most cases requires no particular modification aside from that involved in securing very thin sections.

Recent modifications of the paraffin method make possible its application to insect eggs (see p. 40).

## X. Smear Methods.

Of particular value in cytological investigations, especially in regard to germ cells, are the so-called smear methods. The general effect of the use of any of these is to spread out the cells in a thin layer on the glass slip or cover. By varying the pressure there are produced regions in which cells present distinct degrees of compression, extending even to the bursting of the cell and the liberation of its elements. Except in the

latter case, the entire cell is preserved so that certain inaccuracies, which may be noticed in the study of serial sections passing through an individual cell, are avoided. Smear methods, therefore, are particularly valuable in the study of chromosome complexes where numerical relations are of primary importance. As was indicated in Part 1, it is not wise to rely entirely upon the appearances found in smear preparations and they should always be carefully checked up with similar conditions in sections. Judiciously employed, smear methods are of the very greatest value and are always to be resorted to when practicable. Until within recent years this method has found little application in the study of plant cells. Recently, however, Taylor and Kaufmann, by a simple modification in the method of applying pressure, have been able to achieve valuable results in the study of both somatic and germ cells of plants. The very great value of the method in laying bare details of cell structure, by separating the parts through pressure, is demonstrated. For an account of this application see Chapter IV.

While the smear method is simple in principle, there are difficulties in its application which make it desirable to give somewhat in detail the steps involved. Accordingly the application of the method to the testis of an insect will be described.

First, the organ is exposed by a ventral incision of the body and it is then freed from the surrounding tissues. Previously, appropriate sized cover glasses have been cleaned and are ready for use. In selecting these covers they should be of such a size as to supply space for the entire contents of the testis, or, if this be too large, for an appropriate sized piece of the organ. This is an essential step since if the material is too great some will be lost, whereas if it is too small the pressure and traction may be excessive and destroy many of the cells. The best conditions are provided when the material just fills the space between the covers.

With the material thus placed a slight pressure is applied by means of forceps. This is a delicate portion of the operation and should be judged by the extent to which the follicles are ruptured, permitting the germ cells to separate thinly between the cover glasses. If the testis is from a young animal, the connective tissue is not very resistant and spreading is easily accomplished, whereas if it is from an adult with a greater amount and density of connective tissue the pressure will have to be increased correspondingly. There is also a considerable difference between species in this regard and only experience with a particular material will permit one to operate successfully in every case.

Assuming that the cells have been properly spread, the next operation is to slide the covers apart in the plane of their contact with a fairly rapid and uniform movement. If there is a variation here it will be reflected in the irregularity of the film. The object is to secure a uniform distribution of the cells in a single layer. It is sometimes desirable to proceed one step further in order to rupture



some cells, thus liberating their contents. Especially in the study of the minute details of chromosome structures, such free elements are of particular value.

As soon as the two covers are separated they should at once be inverted upon the fixing fluid so that the film is directly exposed to its action. It is absolutely necessary in most cases to avoid any drying of the cells. Fixation of films may be accomplished by almost any of the common reagents, but, as in other instances, the picro-formol-acetic mixtures and Flemming's fluid commonly give the best results. Since the film is thin, fixation occurs very rapidly and an exposure of a few minutes is sufficient. After fixation the film is treated very much as though it were a section and the processes of staining and mounting are carried out according to directions for sections. For staining it is often of value to have one of the pair of smears stained in iron hematoxylin and the other in Flemming's tricolor when the fixation permits. If large numbers of cover glasses are handled at one time they may be carried through together in racks made for the purpose.

A variation of this process which was introduced by Foot and Strobél for handling Hemipteran male germ cells is carried out as follows:

The testis is seized by a pair of forceps and dragged back and forth in a regular pattern on the surface of a clean glass slip until the entire contents are distributed in a film. This is merely allowed to dry in order to secure fixation and is then stained with Bismarck brown and mounted in balsam. Such a procedure is quite inapplicable to Orthopteran germ cells and to those of many other insects, but works well with the Hemiptera. Still another variation of the smear method was introduced by Foot and Strobél<sup>2</sup> for the study of the eggs of Allolobophora. In the operation of this method the individual eggs are isolated in small drops of water on a clean glass slip and each of them is pricked with a needle and the contents allowed to flow out and distribute themselves in the small quantity of water present. By simple evaporation of these small droplets the material is fixed and is then ready for staining and mounting. Both of the methods of Foot and Strobél are somewhat unusual and are particularly adapted to the materials which they employed. The results obtained, are so good that they warrant a trial in other cases.

### XI. The Aceto-carmine Method

A method very valuable for rapid results is one employing Schneider's aceto-carmine, by means of which the cells are fixed and stained simultaneously. This was utilized to great advantage by Miss Stevens in an extensive series of studies on germ cells of insects. It has the disadvantage of causing extensive swelling of the chromosomes accompanied by much internal distortion of detail and the results lack permanence. If it is desired to preserve the specimens for a limited time this may be ac-

<sup>2</sup> Foot, K., and Strobél, E. C. *Am. J. Anat.*, 4: No. 2, 1905.

complished by ringing a cover glass with some cement which will prevent evaporation. In recent years Belling has somewhat modified this method and has made much use of it in his studies on plant cells. For further discussion of this application of the method see Chapter iv.

For the study of the chromosomes in *Sciara*, Schmuck and Metz use the following method:

- (1) Fix the eggs in Carnoy (equal parts of chloroform, absolute alcohol and glacial acetic acid) one-half to two hours.
- (2) Hydrate through descending grades of alcohol, one-half to one hour in each.
- (3) Stain with Feulgen (see p. 629) eight to ten minutes in fuchsin-sulphurous acid.
- (4) Dehydrate, five minutes in each alcohol to 85 per cent.
- (5) Clear in xylol.
- (6) Mount in balsam.
- (7) Move the eggs into desired positions by pressure of the coverglass.

### C. SPECIAL METHODS<sup>3</sup>

#### I. The Mitochondria

1. **Examination of Living Cells Unstained.** Mitochondria may be studied in living cells teased out in serum or physiological salt solution without the addition of any stain by direct illumination or with the dark field. Their appearance by the latter method is beautifully illustrated by Strangeways and Canti (1927).<sup>4</sup> In animals they are perhaps best seen in the acinous cells of the pancreas where they are of unusually large size and may be recognized by their filamentous shape. Favorable plant material is afforded by pumpkin hairs, for the examination of which during life Maximow's (1913)<sup>5</sup> paper will serve as a guide. Mitochondria may also be readily studied by direct and oblique illumination in the living and growing cells of tissue cultures.

2. **Supravital Staining.** The most satisfactory dyes are Janus green B, Janus blue, Janus black I, and diethylsafranin. The first three are the only known specific stains for mitochondria. Their chemistry is described by Cowdry (1918). They may be applied by immersion or injection. Janus green B is diethylsafranin-azo-dimethyl-aniline chloride.

a. *Immersion.* The best results are obtained with blood (Cowdry, 1914) as follows:

<sup>3</sup> Section on Special Methods by E. V. Cowdry.

<sup>4</sup> Strangeways, T. S. P., and Canti, R. G. *Quart. J. Micr. Sci.*, 71: 1, 1927.

<sup>5</sup> Maximow, A. *Anat. Anz.*, 43: 241, 1913.

Janus green B should be employed in a concentration of about 1:10,000 in 0.85 per cent sodium-chloride solution. A drop should be placed on each of a series of six or more slips. A small amount of freshly drawn blood is then added to the dye and a cover glass is immediately dropped on it. No attempt should be made to mix the blood with the stain before covering.

The preparations should now be examined. Almost immediately one of them will begin to show mitochondria, first in the lymphocytes and later in the granular leucocytes. Soon the mitochondria will be stained in all of them. Under favorable conditions the color will last for several hours. Evaporation may be reduced by putting a ring of vaseline around the edges of the cover glass.

It is difficult in this way to secure a good coloration of mitochondria in nerve cells and most plant cells because the dye penetrates poorly.

*b. Injection.* This method is most satisfactory with the pancreas (Bensley, 1911) and salivary glands, but may be employed with all organs possessed of a rich blood supply. It does not, however, work well with the brain.

The animal is killed and Janus green B is injected into the left ventricle or aorta in a concentration of 1:10,000 of salt solution by gravity pressure. In order to obtain a good penetration the return flow through the inferior or superior vena cava, as the case may be, should be momentarily cut off by artery clamps. After about 10 minutes' perfusion, small portions of the gland may be removed and examined for mitochondria. When the desired intensity of staining has been reached, the entire gland should be placed in salt solution pending examination.

### 3. Fixation and Staining.

*a. Altmann's* (1890) aniline-fuchsin-picric acid method (slightly modified):

(1) Fix small fragments, not more than 2 mm. thick, in 5 per cent potassium bichromate 10 c.c. and 2 per cent osmic acid 10 c.c., twenty-four hours.

(2) Wash in water one hour.

(3) Dehydrate in 50, 70, 95 per cent, and absolute alcohol twelve to twenty-four hours each.

(4) Half absolute alcohol and xylol, three hours.

(5) Xylol, three hours.

(6) 60°C. paraffin three hours. Imbed. Cut sections  $3\mu$  to  $4\mu$ , and fix to slips by albumen-water method.

(7) Pass down through toluol, 95, 70, and 50 per cent alcohol, about thirty seconds each, to aq. dest. in staining jars.

(8) Stain for six minutes in Altmann's aniline fuchsin (aniline water 100 c.c., acid fuchsin 20 gm.). The stain may be poured onto the slide and the whole gently heated over a spirit lamp.

(9) Blot and differentiate by carefully flooding the section with a mixture of

1 part of sat. alc. solution of picric acid and 2 parts of aq. dest., added with a pipette. During this operation the color can be best seen against a white background.

(10) Rinse rapidly in 95 per cent alcohol. Pass through several changes of absolute into xylol and mount in balsam.

In this way the mitochondria are stained a beautiful crimson color against a bright yellow cytoplasm. It is the oldest and in many respects the best of mitochondrial methods, but it has two disadvantages—the fixative penetrates badly and the colors fade rapidly. Accordingly, neutral balsam or cedar oil adapted for immersion objectives should be used, the specimens should not be exposed to direct sunlight or to heat, and they should be kept in a dry place.

*Bensley* proceeds as follows:

(1) 2.5 per cent potassium bichromate, 8 c.c., 2 per cent osmic acid 2 c.c. glacial acetic acid 1 drop, twenty-four hours.

(2) Wash, dehydrate, clear, and imbed (p. 16), except that bergamot oil is substituted for xylol.

(3) Pass section down to water.

(4) Dip in 1 per cent potassium permanganate about one minute.

(5) Rinse in 5 per cent oxalic acid same time and wash in water.

(6) Stain with aniline fuchsin as indicated.

(7) Differentiate in a 1 per cent aqueous solution of methyl green.

(8) Rinse rapidly in 95 per cent alcohol. Pass through several changes of absolute into xylol and mount in balsam.

The use of permanganate and oxalic acid corrects excessive mordanting with the osmic acid and bichromate. It may sometimes be dispensed with. The methyl green, which was first used in this way by Galeotti, is a much finer contrast stain than the picric acid and is also more permanent. The precautions already mentioned against fading should be observed.

A second modification (Cowdry, 1918) may be given:

(1) Regaud's mixture (3 per cent potassium bichromate 20 c.c. and formalin 5 c.c.). The commercial formalin may profitably be neutralized by saturation with magnesium carbonate. The mixture may be applied by immersion or injection, the latter being recommended for large objects. It should be changed every day for four days and be kept in an ice-box (though this is not essential). Mordant for eight days in 3 per cent potassium bichromate, changing every second day.

(2) Wash in running water over night.

(3) Dehydrate, clear, and imbed as indicated (p. 16).

(4) Pass slides to water as indicated.

(5) 1 per cent potassium permanganate thirty seconds, but time must be determined experimentally.

(6) 5 per cent oxalic acid thirty seconds. Steps (5) and (6) may usually be dispensed with.

(7) Rinse in several changes of distilled water about one minute. Incomplete washing prevents staining with fuchsin.

(8) Stain in Altmann's aniline fuchsin made up as follows: Make a saturated solution of aniline oil in distilled water by shaking the two together. Filter and add 10 gm. of acid fuchsin (Duesberg) to 100 c.c. of the filtrate. The stain should be ready to use in about twenty-four hours. It goes bad in about a month. To stain, dry the slide with a towel, except the small area to which the sections are attached; cover the sections with the stain and heat over a spirit lamp until fumes, smelling strongly of aniline oil, come off; allow to cool; let the stain remain on the sections about six minutes; return the stain to the bottle.

(9) Dry off most of the stain with a towel and rinse in distilled water, so that the only remaining stain is in the sections. If a large amount of the stain is left it will form a troublesome precipitate with methyl green; on the other hand, if too much stain is removed the coloration of the mitochondria will be faint.

(10) Allow a little 1 per cent methyl green, added with a pipette, to flow over the sections, holding the slide over a piece of white paper, so that the colors may be seen. Apply the methyl green for about five seconds at first and modify as required. This is the crucial point of the method.

(11) Drain off excess of stain, plunge into 95 per cent alcohol for a second or two. Then rinse in absolute alcohol, clear in toluol, and mount in balsam.

(a) The methyl green may remove all the fuchsin, even when applied only for a short time. This is due to incomplete mordanting of the mitochondria by the chrome salts in the fixative. It may be avoided by omitting steps (5) and (6), or by treating the sections with 2 per cent potassium bichromate for a few seconds just before staining (as advised by Bensley). The action of the permanganate and oxalic is to remove the bichromate.

(b) The fuchsin may stain so intensely that the methyl green removes it imperfectly or not at all. This, on the contrary, is due to too much mordanting. It may be corrected by prolonging steps (5) and (6).

(c) Sometimes, after obtaining a good differentiation, the methyl green is washed out before the slide is placed in toluol, in which event omit the 95 per cent and pass to absolute direct.

This fixative is a good penetrator, in which respect it is much superior to Altmann's fluid or Bensley's mixture. The staining is satisfactory and uniform. Excellent results are obtained with plants as well as with animals. It is recommended for pathological examinations.

*b. Benda's (1901) crystal violet alizarin method:*

- (1) Flemming's fluid, eight days.
- (2) Wash one hour, half pyroligneous acid and 1 per cent chromic acid, twenty-four hours.
- (3) Two per cent potassium bichromate, twenty-four hours.
- (4) Wash in running water twenty-four hours, dehydrate, and imbed in paraffin.
- (5) Mordant sections in 4 per cent iron alum, twenty-four hours.
- (6) Rinse in water and bring into an amber-colored solution of sodium sulph-alizarinate, made by adding a saturated alcoholic solution to water, twenty-four hours.
- (7) Blot with filter-paper and stain in equal parts of crystal violet solution and water. The crystal violet solution made of sat. sol. crystal violet in 70 per cent alcohol 1 volume, alcohol 1 volume, and aniline water 2 volumes.
- (8) The solution is warmed until the vapor arises and then allowed to cool for five minutes.
- (9) Blot, and immerse in 30 per cent acetic acid one minute.
- (10) Blot, plunge in absolute alcohol until but little more stain comes off, clear in xylol, and mount in balsam.

A useful modification is given by Meves and Duesberg (1908). Successful Benda preparations are excellent. The mitochondria are stained a deep violet color against a rose background. They are also much more permanent than Altmann preparations. Unfortunately the method is long, tedious, and difficult. It has been much employed in the study of spermatogenesis.

*c. Champy-Kull's* (1913) aniline-fuchsin, toluidin blue and aurantia. This is a modification of the methods of Altmann and Benda:

- (1) Champy's fluid:

3 per cent potassium bichromate .....	7 c.c.
1 per cent chromic acid .....	7 c.c.
2 per cent osmic acid .....	4 c.c.
Twenty-four hours.	

- (2) Wash in aq. dest., then a mixture of 1 part acetic acid pyrolignosum rect. and 2 parts 1 per cent chromic acid, twenty hours.
- (3) Wash in aq. dest. thirty minutes and mordant in 3 per cent potassium bichromate, three days.
- (4) Wash in running water twenty-four hours, dehydrate, clear, imbed and section.
- (5) Altmann's aniline fuchsin (10 gm. acid fuchsin to 100 c.c. aniline water) heating quietly over flame.
- (6) Allow to cool six minutes, pour off stain, wash rapidly in aq. dest.
- (7) Counterstain with 0.5 per cent toluidin blue one to two minutes. Rinse in aq. dest.

- (8) 0.5 per cent aurantia in 70 per cent alcohol, twenty to forty seconds.
- (9) Differentiate in 95 per cent alcohol, dehydrate, clear and mount.

The nuclei are colored blue, the mitochondria red, the ground substance greenish yellow. It is especially recommended by Gatenby for invertebrates.

*d. Regaud* (1910) has used the iron-hematoxylin method of Heidenhain after a large variety of fixatives, the best of which is his formalin and bichromate mixture.

- (1) 3 per cent potassium bichromate 20 c.c., formalin 5 c.c., for four days, changing every day.
- (2) Mordant in 3 per cent bichromate for seven days, changing every second day.
- (3) Wash in running water twenty-four hours, dehydrate, clear, imbed, and section as indicated.
- (4) Pass sections down to water as indicated.
- (5) Mordant in 5 per cent iron alum at 35°C. for twenty-four hours. Rinse in aq. dest.
- (6) Stain for twenty-four hours in hematoxylin made up as follows: Dissolve 1 gm. pure crystals of hematoxylin in 10 c.c. of absolute alcohol and add 10 c.c. of glycerin and 80 c.c. of distilled water. A few weeks should be allowed for the stain to ripen. When ready for use it can be employed over and over again for about ten times. The traces of iron alum added to the stain are helpful. The crucial point in the technique is this passing from the mordant to hematoxylin. The slides must be rinsed in *distilled* water, otherwise the iron alum will form a dense black precipitate in the stain. On the other hand, if they are rinsed too much, all the iron alum mordant will be removed. It is necessary to strike the happy mean in which a darkening of the hematoxylin alone occurs. It is always difficult to get good hematoxylin, and it is best to keep on hand a ripe alcoholic solution.
- (7) Differentiate in 5 per cent iron alum under microscope, wash in tap water half an hour, dehydrate, clear and mount.

This is the most permanent as well as the simplest of all mitochondrial stains. It may be used in the damp climates of most of our marine biological laboratories, where the Altmann method and its modifications are useless. It is advised that the beginner try it with an organ like the pancreas. Unfortunately the fixation rarely gives good results with embryonic tissues; for these the older osmic acid-containing fixatives are best adapted. It is often possible to make use of material fixed in the usual way with formalin by starting out with step (2). Moreover, the preparations can be counterstained in a variety of ways (Cowdry, 1916). The coloration is less specific than that obtained by modifications of the Altmann method. Many cytoplasmic granulations which are not mitochondria are, like the mitochondria, colored blue-black.

*e. Murray's method for mitochondria and bacteria.* This is a modification of the preceding technique.

(1) Formol-Müller. (Potassium bichromate 2.5 gm., sodium sulphate 1 gm., distilled water 100 c.c., formalin 10 c.c.) over night.

(2) Mordant in Müller's fluid two to seven days.

(3) Wash in running water, dehydrate, clear and imbed in paraffin as indicated. Cut sections 5 $\mu$ .

(4) Remove paraffin, pass to water.

(5) 3.5 per cent iron alum at 50°C., fifteen minutes.

(6) 0.5 per cent aq. hematoxylin at 50°C., fifteen minutes.

(7) Differentiate with similar iron alum solution. Dehydrate, clear and mount in balsam. In this way both mitochondria and bacteria are colored bluish black. If the sections are decolorized with 0.5 per cent hydrochloric acid in 70 per cent alcohol the bacteria alone remain colored.

Bacteria are often well stained also by the various modifications of Altmann's method. When the differentiator is methyl green they are colored green, when toluidin or methylene blue, they are blue; while the mitochondria are stained crimson with the fuchsin. The root nodules of clover afford satisfactory material because they are easily obtained and invariably contain readily stainable intracellular bacteria and mitochondria.

*f. Dubreuil's (1913) iron hematoxylin method for blood cells:*

(1) Take up the fluid to be examined in a pipette containing several times its volume of 0.5 to 1 per cent osmic acid. Transfer to a centrifuge tube. A good fixation is obtained in about an hour. Then add aq. dest., centrifuge, and decant. The blood cells remaining are shaken up with absolute alcohol and then passed into a weak solution of celloidin. A drop is allowed to spread on a slide, which, before complete desiccation, is plunged into 80 per cent alcohol.

(2) The mitochondria in the cells are then stained with iron hematoxylin, as indicated above.

*g. Bensley's copper chrome hematoxylin method.* By this method the mitochondria are colored blue against a yellowish brown background.

(1) Either Altmann's osmic bichromate mixture (p. 266) or in Bensley's acetic osmic-bichromate fluid (p. 267), twelve to twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Pass down to water. Saturated aqueous copper acetate, five minutes.

(4) Wash in several changes distilled water, one minute, 0.5 per cent hematoxylin, one minute. If the copper acetate has not been sufficiently washed out, a black precipitate forms in the hematoxylin. The hematoxylin should be well ripened. It may be obtained by dilution down from a 10 per cent alcoholic stock solution.

(5) Rinse in aq. dest. 5 per cent neutral potassium chromate, one minute.



The sections should turn a dark blue-black color. If they are only a light-blue shade, rinse in aq. dest., place again in the copper acetate, and carry through as just described several times until no increase in color results.

(6) Wash in aq. dest. and return for a few seconds to the copper acetate in order to convert all the dye into the copper lake.

(7) Wash in aq. dest.

(8) Differentiate under the microscope in Weigert's borax-ferricyanide mixture (borax 1 gm., potassium ferricyanide 1.25 gm., and aq. dest. 100 c.c.) diluted with 2 volumes of aq. dest.

(9) Wash six to eight hours in tap water.

(10) Dehydrate, clear, and mount in balsam.

*h. Bensley's (1911) neutral safranin method:*

(1) 2.5 per cent potassium bichromate 100 c.c., mercuric chloride 5 gm., for twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Preparation of stain: Add slowly sat. aq. sol. of the color acid, acid violet, to sat. aq. sol. of the color-base, safranin O, contained in a flask until a precipitate no longer forms. The point of neutralization may be roughly determined by dropping a little of the mixture on filter-paper from time to time until the outside red ring of safranin disappears and the whole blot takes on a neutral color. Filter. The filtrate should be as nearly as possible colorless. Dry the precipitate on filter-paper for twelve hours, collect it, and make a saturated solution of it in absolute alcohol.

(4) Pass sections down through two changes of toluol and absolute alcohol in order to remove all traces of paraffin or toluol, which might interfere with the staining. Then through 95, 70, and 50 per cent to aq. dest. (Chrome- and osmium-fixed material must be bleached in permanganate and oxalic acid, and sublimate-fixed tissues must be treated with Lugol's iodine solution for about ten seconds and washed in aq. dest.).

(5) Dilute the alcoholic stock solution of the dye with an equal volume of aq. dest. and stain for from five minutes to two hours.

(6) Blot quickly with several layers of filter paper.

(7) Plunge into pure acetone and pass immediately to toluol without waiting to drain.

(8) Examine under the oil immersion and if necessary differentiate in oil of cloves. If this is not sufficient, the slide, after rinsing in absolute alcohol, may be instantaneously flooded with 95 per cent alcohol, and then passed back through absolute alcohol to toluol.

(9) Wash in two changes of toluol and mount in balsam.

Working on the same principle, a number of stains can be made up for mitochondria (Cowdry, 1913). Note also Bensley's neutral gentian method. These methods were devised by Bensley chiefly to aid in the study of the mitochondria and other cytoplasmic constituents in the pancreas.

i. *Bensley's* brazilin-wasserblau method for the mitochondria and secretion antecedents of the thyroid gland (1916).

(1) Zenker's fluid, less acetic acid, plus 10 per cent formalin, twenty-four hours.

(2) Wash, dehydrate, clear, imbed, and section.

(3) Pass down to water.

(4) Iodize with Lugol's solution, thirty seconds.

(5) Stain in following solution several hours: Phosphotungstic acid, 1 gm.; aq. dest., 100 c.c.; brazilin, 0.05 gm. The brazilin is first dissolved in a small quantity of distilled water by the aid of heat and added to the phosphotungstic acid solution. Ripening may be accelerated by the addition of 0.4 c.c. of hydrogen peroxide, or of a few drops of a solution of soluble molybdic acid. The solution deteriorates with age and should not be used after three days.

(6) Rinse in aq. dest. and place for one to five minutes in phosphotungstic acid, 1 gm. wasserblau, 0.2 gm.; aq. dest., 100 c.c.

(7) Wash rapidly in water, dehydrate in absolute alcohol, clear in foluol, and mount in balsam.

j. *Meve's* (1905) new Victoria green method: This method is intended for red blood cells which are simply stained in the fresh condition by the addition of a 4 per cent iodic-acid solution to which a small quantity of new Victoria green (malachite green) has been added.

k. *The methods of silver reduction* employed by many Italian investigators are essentially modifications of the original method of Golgi (p. 446). They undoubtedly reveal mitochondria in most cases, but one would hesitate to attribute any high degree of specificity to them.

4. **Experimental Error in Mitochondrial Technique.** Mechanical injury to the cells by the use of forceps during removal, before fixation, must be avoided. Allowing a surface film of the tissue to dry in air as it stands on the autopsy table will alter the whole appearance of the contained mitochondria. Osmotic changes are likewise harmful. Merely keeping the tissue in salt solution is detrimental. If the tissues cannot be fixed absolutely fresh, they should be set aside in a cool place and the surface layers should be removed with a razor just before preservation.

The various ingredients of the fixations have different powers of penetration. In respect to the most superficial cells they act simultaneously and give good preservation. As one passes inward their influence is successive and various types of artifact are often produced.

Before reaching any conclusions as to mitochondrial alterations in experimental conditions, it is essential to make sure that all the mitochondria have been preserved and that they retain the form exhibited during life. The number of mitochondria may appear to be reduced after faulty fixation, incomplete mordanting and excessive differentiation

of the stain. Mitochondria are never increased in amount through technical errors. The most common change in the mitochondria brought about by fixation is a segmentation or rounding up of rods and filaments into spherules. Consequently the experimenter will wish to assure himself that this alteration is not taking place by comparison with living unstained and supravitaly colored cells. The reverse alteration, of a lengthening of the mitochondria, never results from mistakes in technique.

Special methods for the quantitative estimation of mitochondria have recently been devised by (Du Nöij and Cowdry, 1927,<sup>6</sup> and Cowdry and Covell, 1927<sup>7</sup>).

## II. The Golgi Apparatus (Reticular Apparatus Bennennetz<sup>8</sup>)

While there is so little agreement as to just what the Golgi apparatus is, it is difficult to describe the technique for its demonstration. What may, however, be regarded as the "type structure" was first revealed by Golgi (1898) in nerve cells through fixation in a mixture containing potassium bichromate and osmic acid followed by impregnation with silver. The apparatus appears jet black against a yellowish background. It is a conspicuous structure consisting of an intricate network of anastomosing strands. This network may closely envelop the nucleus, be concentrated to one side of it, or else be scattered rather diffusely throughout the cytoplasm.

In 1902 Kopsch showed that the same material can be blackened by prolonged treatment with 2 per cent osmic acid. On this affinity for both silver and osmium all the modern methods for revealing the Golgi apparatus are based. Few cytological reactions are more fickle and inconstant, but, when after many attempts the technique is successful, convincing and very beautiful preparations result.

Unlike the mitochondria, the Golgi apparatus cannot be studied unstained or supravitaly colored in the living cell with any degree of satisfaction except perhaps in some plants. For a summary of advances in this direction, and for much original work, including the supravital coloration of the apparatus with neutral red in *saprolegnia*, see the monograph of Guilliermond.<sup>9</sup> Parat and his associates, in a brilliant series of studies, have advanced the view that the material which we

<sup>6</sup> Du Nöij, P. L., and Cowdry, E. V. *Anat. Record*, 34: 313, 1927.

<sup>7</sup> Cowdry, E. V., and Covell, W. P. *Anat. Record*, vol. 34: 1927.

<sup>8</sup> For a discussion of the relation of the Canalicular apparatus, *Trophospongium Säftkanälchen*, etc., to the Golgi apparatus see: Cowdry, E. V., (ed.) *General Cytology*, Chicago, 1924.

recognize as the Golgi apparatus in animal cells which have been impregnated with silver or with osmium, is represented in the living cell by droplets which may be colored with neutral red. We await a final proof of this hypothesis with eagerness.

With both silver and osmium methods considerable experimentation is necessary in order to obtain the best results. The factors to be varied are principally the composition of the fixative and impregnating substance and the time during which they are allowed to act. During impregnation it is always advisable to keep the tissues in the dark and instructions as to temperature requirements should be carefully followed. When either the silver nitrate or osmic acid becomes blackened it should be renewed. It is important for the beginner to start with the most favorable material. The spinal ganglion cells of young mammals such as the rabbit are perhaps the best for this purpose. The acinous cells of the pancreas are also recommended but are somewhat more difficult to handle. All of the methods of impregnation outlined below frequently bring to light the mitochondria also.

### 1. Silver Methods.

a. *Cajal's* (1912) uranium nitrate silver method.<sup>10</sup> This is one of many methods devised by Cajal. It is recommended for embryos and young animals.

(1) Uranium nitrate 1 gm., formalin 15 c.c., and aq. dest. 100 c.c. eight to twenty-four hours.

(2) Wash quickly in aq. dest.

(3) 1.5 per cent silver nitrate twenty-four to forty-eight hours.

(4) Rinse in aq. dest.

(5) Hydrochinon 2 gm., formalin 6 c.c., aq. dest. 100 c.c., anhydrous sodium sulphate 0.15 gm., twelve hours.

(6) Wash in aq. dest., dehydrate quickly, clear, imbed, and section.

b. *Da Fano's* (1920)<sup>11</sup> cobalt nitrate silver method. Here the uranium nitrate is replaced by cobalt nitrate. In other respects the technique is similar. De Fano has, however, so carefully attempted to control troublesome experimental conditions that the various steps are given in detail.

(1) Fix in cobalt nitrate 1 gm., aq. dest. 100 c.c., formalin 15 c.c. six to eight hours. The formalin need not be neutralized unless it is strongly acid. In the case of embryos and delicate tissues, when shrinkage is to be feared, reduce the

<sup>9</sup> Guilliermond, A. *Arch. d'Anat. Micr.*, 23: 1, 1927.

<sup>10</sup> Many useful hints are given by Carleton, H. H. *J. Roy. Micr. Soc.*, p. 321, 1919.

<sup>11</sup> Da Fano, *J. Roy. Micr. Sci.*, p. 157, 1920.

formalin to as little as 6 c.c. With cartilage and small pieces less than 3 mm. thick, like the organs of mice, shorten the time of fixation to three to four hours. Hollow organs, such as the stomach and intestine, are better with the fixing fluid for one hour and then cut into pieces of convenient size and shape. For the spinal cord, cerebellum and cerebrum of adults, eight to ten hours is recommended, but fixation should never exceed twenty-four hours. In the case of the testicle, he advises injection of the fixative through the aorta and then immersion in it.

(2) Wash quickly in aq. dest. and impregnate in 1.5 per cent silver nitrate twenty-four to forty-eight hours. The concentration of silver nitrate should be reduced to 1 per cent for very small fragments easily permeable, and be increased to 2 per cent for tissues containing much fat and for the spinal cord. Impregnation is effected at room temperature in a majority of cases. When difficulty is experienced in impregnation the use of an incubator at 36° to 37°C. is advised.

(3) Wash rapidly in aq. dest. and cut down the tissues again to a thickness of 2 mm. or less.

(4) Reduce in Cajal's mixture, above mentioned, twelve to twenty-four hours.

(5) Wash in aq. dest. one-half hour. Cut with a freezing microtome or imbed in paraffin. The Golgi apparatus should be colored dark brown or black against a yellow background. The preparations may be made more permanent by gold toning.

(6) Pass to water. Then 0.1 to 0.2 per cent gold chloride, two hours.

(7) Counterstain with alum carmine, dehydrate, clear and mount.

## 2. Osmium Methods.

*a. Kopsch's Method.* Immersion of small pieces of tissues in 2 per cent osmic acid for eight to sixteen days often brings to light the Golgi apparatus but there is considerable shrinkage and the tissues become rather brittle.

*b. Sjoval's (1905) modification:*

(1) 10 per cent formalin, eight hours.

(2) Wash in aq. dest.

(3) 2 per cent osmic acid at 35°C., two days.

(4) Dehydrate, clear, imbed.

*c. Hirschler's (1918) modification:*

(1) Saturated aqueous mercuric chloride 10 c.c., 2 per cent osmic 10 c.c., at room temperature one to three hours.

(2) Wash in running water then in aq. dest., one-half hour.

(3) Two per cent osmic acid at 25°C., twelve to sixteen days.

(4) Wash twenty-four hours in running water, dehydrate, clear in chloroform and imbed.

*d. Kolatchew's method (Nassonov, 1924):*

(1) 3 per cent potassium bichromate 10 c.c., 1 per cent chromic acid 10 c.c., and 2 per cent osmic acid 5 c.c., twenty-four hours.

- (2) Wash in running water twenty-four hours.
- (3) 2 per cent osmic acid 40°C., eight hours, three to five days at 35°C.
- (4) Wash in aq. dest., dehydrate, clear, and imbed.

*e. Weigert's Mann-Kopsch method as modified by Gatenby:*<sup>12</sup>

- (1) Mann osmio-sublimate mixture (sat. aq. corrosive sublimate in salt sol., 10 c.c., 1 per cent osmic acid, 10 c.c.) one quarter to three hours or more.
- (2) Wash in aq. dest. fifteen to thirty minutes.
- (3) 2 per cent osmic acid, room temperature ten to fourteen days.
- (4) Wash in running water two hours or more.
- (5) Dehydrate, clear, and imbed.

The Golgi apparatus is blackened and the mitochondria and ground substance are colored reddish brown. Gatenby suggests as subsequent treatment:

(a) "The blackening may be extracted step by step in turpentine, and the appearance of the cell granules studied at intervals.

(b) "If the mitochondria are not stained black by the  $\text{OsO}_4$ , one may proceed directly to the Altmann method (but preferably after cautious treatment in 0.125 per cent permanganate of potash).

(c) "The nuclear structures may be stained in safranin, crystal violet, or acid fuchsin. The sections are brought down to distilled water and transferred to watery solutions of the dye. A few minutes generally suffice to stain the nuclei."

Many suggestive experiments have been made with the Mann-Kopsch method by Ludford.<sup>13</sup> He has found that the time of fixation bears out little relation to the degree of impregnation with osmic acid, and that better impregnation is obtained when the temperature of the osmic bath is increased above that of the room. With progressive rise in temperature the following sequence of changes was observed:

(a) "The apparatus appears first as granules or faint rodlets.

(b) "The rodlets become thicker and the cytoplasm commences to shrink.

(c) "The rodlets appear to be anastomosed to form a network, and the ground substance becomes coarsely granular.

(d) "The whole of the Golgi apparatus is impregnated deep black, and the ground cytoplasm is distorted so as to give the appearance of a tangled network, or reticular structure, and there is considerable non-specific reduction of the osmic acid.

(e) "Thereafter, the cell becomes more and more deeply osmicated, until the cytoplasm appears homogeneously black."

<sup>12</sup> Lee's *Microtomist's Vade-mecum*. Ed. 9. Ed. by Gatenby, J. B., and Cowdry, E. V., London, 1928.

<sup>13</sup> Ludford, R. J., *J. Roy. Micr. Soc.*, p. 269, 1924.

Cells which have thus become very black may be bleached by dilute solutions of potassium permanganate, by hydrogen peroxide and by nascent chloride. Ludford prefers the last. In view of these experiments he says the "Golgi apparatus is that region of the cytoplasm of cells which brings about the reduction of osmium tetroxide at a lower temperature, or in a shorter time, than is required to produce a total blackening of the cell."

**3. Experimental Error in Revealing the Golgi Apparatus.** Some investigators prefer to impregnate the Golgi apparatus with silver and others with osmium. Which is the least open to objection it is difficult to say. The precautions to be observed are in many respects similar to those mentioned under the heading of "Mitochondria," p. 265. The influence of variations in temperature has not been so carefully studied with silver as with osmium. Before placing any reliance in the Golgi apparatus as an indicator of cellular activity it is essential to make sure that the technique being used brings to light all the Golgi apparatus, not only a part of it. The surface and volume of this peculiar structure have recently been measured quantitatively by means of a special technique in spinal ganglion cells.<sup>14</sup>

<sup>14</sup> Covell, W. P. *Anat. Record*, 35: 149, 1927.

## CHAPTER VI

### EMBRYOLOGICAL METHODS

C. E. McCLUNG, EZRA ALLEN AND RUTH McCLUNG JONES

Introduction 279. Securing embryological material 279. Study of living material in normal and isotonic media 281. Prepared material 282.

#### I. Introduction

The range of embryological methods is extensive and their character varied, so that in a book of this sort it would be impossible to treat them exhaustively. Especially is this true if the practices of experimental embryology are involved. The only consideration that the latter will receive will be in connection with the treatment of micromanipulations. The main emphasis here will be placed upon the technique for fixed and sectioned material.

#### II. Securing Embryological Material

Unlike other materials, those used in embryological work may not be obtainable just when wanted, so that it becomes necessary to take into consideration the seasonal occurrence of developmental stages in animals and to follow the rule of getting the material when it is available rather than when it is wanted. Because of the large numbers of animal forms and their variable embryological behavior it will be possible here to consider only types and these very briefly. If one desires information with regard to the various forms available for embryological studies he should consult works devoted to comparative embryology. It will be sufficient here to mention that many aquatic invertebrate forms, both fresh water and marine, offer convenient sources of material. Certain snails may be kept in aquaria and will there provide at intervals batches of eggs which may be traced through the various developmental processes. Fish often are convenient for this purpose. The eggs are stripped from the female and the milt from the male, and after fertilization the successive steps of cleavage and somatogenesis may be obtained.

Amphibian material is usually available only in the spring, but if secured at this time may be obtained in large quantities. Frogs and toads are most often the source of this material, but certain Urodeles also serve. Axolotls are conveniently kept in aquaria and in the spring lay



a number of batches of eggs which are very excellent embryological material.

One of the most extensively studied embryological forms is the chick. It has certain advantages, one of which is the ease with which material may be obtained. All stages of development may be secured by timing the periods of incubation, either under a hen or in an incubator. Mammalian material is very difficult to handle although its source is more continuous and convenient than any other. The commonest forms utilized for this purpose are the mouse, rat, rabbit and pig.

Knowing the reproductive cycle of any animal it is not a matter of great difficulty to obtain a complete series of embryological stages by appropriate timing. In the case of the pig the most convenient method is to go to an abattoir and there secure uteri, collecting a large number of stages which later may be measured and sorted. Because of its availability in this manner the pig has been much used, although it has certain structures which are unusually developed. Later stages of development are not hard to obtain but the early ones offer many more difficulties. As a means for securing these in the case of the mouse, Allen offers the following directions:

*Mouse Embryos.* Fixation of small mammalian embryos *in situ* frequently results in distortion through contraction of the uterine muscles. The following method has avoided this result in mouse embryos from five to eight days gestation age, the critical period. Remove both horns of the uterus by severing the tubes, the mesometrium, and the vagina. Transfer to Locke's solution in a small dish with paraffin-covered bottom. Stretch the horns and pin fast, ventral side up, pinning through the vagina or bladder, the tubes, and the mesometrium. Under a low power binocular remove the muscle from the exposed surface, at least over each capsule (*decidua capsularis*). This may be done by starting at the vagina and carefully dissecting the muscle as a sheet, or removing it in short strips. The capsules may now partly be freed with ease from the underlying muscle and left attached at the mesometrial end. If carefully done no pressure is exerted upon the capsules. The fixative desired may now be added in small quantities, pipetting it directly upon the capsules while in the Locke's solution. In about fifteen minutes, after several applications of the fixing fluid, the pin may be removed; and if no contraction takes place the horns removed to the pure fixative. Destin's fluid (see formula, Chapter IX, p. 559) has given excellent results for both normal and degenerate embryos, but is not to be relied upon for chromosome fixation. Fix from two to eight days, wash for twenty-four hours, and run to 50 per cent alcohol by gradual steps. Again pin fast and dissect the capsules sufficiently to remove one-half of the capsular tissue, watching for the appearance of the embryo. When it is uncovered, it may either be removed and further dehydrated for sectioning, or left in position and the capsule and embryo removed from the muscle and prepared for sectioning together. By staining *in toto* with alcohol cochineal, orientation of the embryo in either case is simple:

the weak stain is readily removed from the sections by acid alcohol. If desired to treat the embryos differently, the capsules may be severed from each other after the muscle has been removed and placed in different fixatives. Bouin's and B-15 are both good fluids for preserving the capsules and embryos, but they render the decidua capsularis a little tougher than does Destin's fixative. If used, the duration of fixation should be as brief as possible, especially if dissection of the capsule is proposed. The chrom-acetic-formol fluid gives a beautifully white embryo and surrounding tissue. Mouse embryos younger than six days of gestation are too small to be seen under the binocular, and their capsules should not be dissected but sectioned whole. Capsules of six to eight day embryos may be trimmed on each side after the embryo is uncovered, thus avoiding many superfluous sections. This trimming should be done before passing to the clearing fluid, but not before they have been in 70 per cent alcohol long enough to harden fairly well.

*Mouse Uteri for Blood Vessels.* For this purpose, either ligate the blood vessels before removing the uterus from the body, or stretch the two horns in the body cavity and cover with Locke's solution. To this add the mixture previously described (p. 249) as B-20. After the horns have stiffened, remove them to the pure fixative and let remain an hour. The fixative may be either warm or at room temperature. Carry to 70 per cent alcohol by gradual steps. When thus treated, the blood tends to retain its color for some time and the vessels stand out against the yellow color of the rest of the tissue.

### III. Study of Living Material in Normal and Isotonic Media

In embryological work it is fortunately the case that the entire history of a single organism may be continuously observed under appropriate conditions. The normal picture of development thus secured is extremely helpful in the interpretation of sectioned material later, and wherever possible should always be sought. The technical processes involved here are extremely simple and require no elaboration. However, when it comes to the experimental modification of normal processes we enter a field of great difficulty and diversity.

Much of the early development of a chick embryo may be observed by producing a window in the shell of the egg. This is best accomplished by using a hard rubber ring of about three quarters of an inch in diameter. The outline of this is marked on the side of the shell with a pencil and within this the shell is thinned by means of a file. The rubber ring is then cemented onto the shell by the use of shellac or a collodion solution. After this is hardened the shell and membrane within the ring are removed and the space filled up with albumen from another egg. Over this is placed a cover glass which soon becomes firmly attached by the drying of the excess albumen. The egg is then put into an incubator

and by turning the window to the upper side of the egg the embryo may be observed at any time and its development traced.

There are two general means for carrying out experimental modifications. The first is by chemical agents and the second by physical or mechanical methods. Because of the great diversity of chemical modifications of development and their special character it is not expedient to discuss them at length here, and those interested are referred to such works as those of Jacques Loeb and others. In recent years mechanical methods for modifying embryological processes have been highly developed and have served to advance our knowledge of normal processes materially. These also are of extreme diversity but because they have not been so fully published the methods will be discussed somewhat at length by Chambers in the chapter on fresh material.

#### IV. Prepared Material

**1. General Character of Processes.** The methods employed for embryological study do not differ materially from similar ones in cytological and histological work. The same fixatives that produce good results on cells and tissues also act best when the entire body of an embryo is to be preserved. Likewise the methods of sectioning in paraffin and collodion are essentially the same as elsewhere employed and do not require repetition here.

*a. Whole Mounts of Fixed Specimens.* It happens, because of the small size of embryos, that they are often studied entire and so we have special methods for mounting and studying whole organisms rather than their parts. Chick embryos are conveniently mounted entire, very much like a stretched preparation. Since these are so often used for embryological studies the method of their preparation will be given fully.

Remove an egg from the incubator, and, holding it in the hand, break the small end by tapping it gently with a blunt instrument. With forceps pick away enough shell to leave an opening of about 15 mm. Pour out as much albumen as possible, cutting the denser portion with scissors if it does not flow readily. With scissors or forceps take away more shell until the yolk is completely exposed, and the broken edge of the shell is on a level with the yolk. The embryo should be uppermost if these operations have been carefully performed. It may be brought to that position if necessary by rotating the yolk with a section lifter.

Place the egg now in a pan of sawdust, sand or planer chips, and pipette on formol-nitric (p. 559) fluid. At least two applications are necessary during the fixing period. When fixation is complete, cut around the sinus terminalis with curved scissors and remove the specimen carefully with a section lifter to a dish of water. Free it from yolk, using a gentle stream of water from a pipette, and

transfer it to a dish of fresh water. At this point remove the vitelline membrane carefully.

Pick up with forceps a square of unglazed paper somewhat larger than the blastodisc. Lift the specimen from the water on this paper, making sure that it lies flat and unwrinkled and that the yolk side is up. Float on Worcester's fluid in a small dish and pipette more of this hardening fluid on the specimen. Rinse in water to which a few drops of iodine have been added, stain in dilute (1 part stain, 10 parts water) alum cochineal, and run up to 70 per cent alcohol.

Differentiate to a clear transparent pink in acid alcohol (5 drops HCl to 10 c.c. 70 per cent alcohol) and return to 70 per cent alcohol. Spread the blastodisc flat on a fresh square of paper and cut through specimen and paper, making a circle, which includes, except in old and large specimens, the sinus terminalis. The cutting causes the tissue to adhere to the paper, so that in subsequent procedures the embryo may be easily handled. Float on 80 per cent alcohol, dehydrate in dioxan, mount in the sandrac medium (p. 40).

The mounting is most easily accomplished if a round coverglass has been prepared in advance by cementing near its edge, equidistant from each other, three strips of celluloid, of such a thickness that the cover will be supported by them and will just clear the chick. Place a 1 x 3 inch slip in the bottom of a narrow, flat dish of dioxan, free the embryo from its paper, maneuver it to the approximate center of the slip, and lift the slip from the dish. Center the chick accurately, place over it the cover and fill the space under the cover with sandrac medium, using a fine pipette. In the course of drying it will probably be necessary to add more sandrac. After cleaning, ring the cover with damar.

By a process of microinjection (Knower, p. 57) such embryos may have their circulatory system filled with India ink and when mounted entire make beautiful preparations. Older embryos may be injected in the same manner. These are not mounted like an ordinary microscopical preparation but are cleared with methyl salicylate and placed in convenient containers for observation.

INFILTRATION SCHEDULE FOR SECTIONED MATERIAL

	Paraffin and Dioxan	Paraffin, 3 Changes
18-24.....	6-18 hours	1 hour
33-40.....	.....	1 ½ hours
48-56.....	.....	2 hours
72-96.....	.....	4 hours

In the study of the developing osseous system, embryos may be rendered transparent by treating them without previous fixation with a 1 per cent solution of potassium hydroxide for twenty-four hours. They are subsequently preserved in glycerin.

*b. Dissected Specimens.* Dissections of embryos, even of small size,

are entirely feasible and, in connection with sections of similar embryos, afford a valuable means of correlation. Heuser has developed very refined methods for such microdissections. He fastens the fixed embryo to a small piece of ground glass with gelatin. After the embryo has become attached it is then dissected under alcohol with the aid of the binocular microscope. In this manner he has been able to trace out even the finest connections of nerves. For some purposes it is better to stain the embryo entire before beginning the dissection and for this purpose alum cochineal is perhaps the best stain.

*c. Sectioned Specimens.* While cleared preparations and dissections are very helpful in reaching an understanding of embryonic structures, final resort in most cases must be made to the study of serial sections. Here, as elsewhere, the preservation of the material in its normal form is of the highest importance. Formerly Zenker's fluid was much recommended as a fixative, but it causes extensive shrinkage and now resort is made almost entirely to the picro-formol-acetic combinations. Picric acid in connection with sulphuric acid in the formula of Kleinenberg has long been used but seems to be inferior to the picro-formol-acetic mixtures (p. 562).

All of the usual precautions with regard to gradual transfer of the specimen from one fluid to the other hold with somewhat added emphasis in the case of embryos. The methods of staining are similar to those used in histological practice with the exception that for most purposes the advantages lie with *in toto* staining. For this purpose no better combination has been evolved than the alum cochineal stain (p. 612).

Excellent preparations may be made if almost the same methods already outlined for whole mounts are followed. Undiluted alum cochineal should be used for staining with no subsequent differentiation. In 70 per cent the specimens are affixed to papers, yolk side up, by cutting, retaining only as much of the extra-embryonic membranes as desired. Dehydration by dioxan is satisfactory. The fixing of the embryo on the paper is of great value in keeping it perfectly flat, since the curling which often occurs otherwise during dehydration makes it difficult to section it accurately in either a frontal or a sagittal plane. The paper is removed before the embryo is sectioned.

It is desirable to adopt a uniform method of arranging embryo sections on a slide. A conventional method for transverse sections is to cut the sections in such a way that after spreading, embryos which have not undergone rotation (i.e. up to about thirty-eight hours) show the notochord on the right hand side of the section when it is viewed through a compound microscope. Correspondingly, older embryos show the noto-

chord in any part of the body which has rotated, below the neural tube. This arrangement can be easily secured in the following way.

The membrane at the cephalic end is cut slantwise with a point on the left some 2 or 3 mm. higher than the right hand side, at the same time the specimen is fixed to the paper. In blocking the specimen it is mounted caudal end toward the block. The block is inserted in the clamp of the microtome so that the point above mentioned is to the left.

TIME SCHEDULE FOR WHOLE MOUNTS

Chicks	Formol-nitric	Water	Worcester	Water	Alum Cochineal	Wash	10, 20, 30 50, 70% alc.	Dioxan, 3 Changes
	min.	min.	min.	min.	hours	min.	min. in each	hours
18-24 hr. .	15	2-5	15	20	18-24	5	10	12-24
33-40.....	20	2-5	20	20	18-24	5	15	12-24
48-56.....	24	2-5	25	30	18-24	10	20	18-24
72-96.....	30	2-5	30	30	18-24	15	25	24-48

For a complete understanding of the structure of a bilaterally symmetrical body such as a vertebrate embryo it is necessary to have embryos of similar stages of development cut in three planes at right angles to each other. Two of these series are longitudinal sections, one passing parallel with the plane which divides the body into right and left halves, and the other at right angles to this. In addition to these two longitudinal sections, one series cut at right angles to them and passing transversely through the body is necessary. These sections, when cut by the paraffin method, come off in a continuous ribbon which is then subdivided and mounted as slides in such manner that by proceeding from left to right successively in one slide after the other, the entire embryo is passed through in order, emerging at the last section on the lower right hand corner of the last slide. The sections are therefore read like the letters in the words of the lines of a printed page. It is obvious that with the necessity for this continuous series of sections, every precaution must be employed in sectioning and in subsequent treatment, to avoid the loss or mutilation of any section or series of sections. Also for ready passage from one section to the other they should be arranged in absolutely straight rows and so placed on the slide that sections at right angles to the rows fall also in lines. A thoroughly good embryological slide will show the sections therefore uniformly spaced and in straight rows.

For small embryos the ordinary size of glass slip,  $1 \times 3$  inches, may

be used, but larger specimens are better mounted upon slips  $1\frac{1}{2} \times 3$  inches, or  $2 \times 3$  inches.

The thickness of sections depends upon the purpose in view. Ordinarily the best thickness is about  $10\mu$ , and it is very desirable to have a microtome which will cut a practically uniform series of sections. This is of particular merit when reconstructions are to be made.

It is always important to secure complete spreading of the sections, but it is of unusual value in the case of embryological sections because if they are to be used for reconstructions any distortion due to unequal or incomplete spreading will seriously interfere with the production of the model.

The experienced worker, having at his command sections of an embryonic stage in the three dimensions, will be able to reconstruct in his mind the general configuration of organs and their relations to each other. However for a permanent record, resort is often had to the process of reconstruction. This consists essentially in making drawings or photographs of sections at regular intervals and from these making transfers to wax plates which are of such a thickness as to correspond in magnification to the two dimensions represented in the section. These wax plates are then cut and piled one upon the other until they reproduce in their essential form the structure of the entire embryo. Of course, due to many technical difficulties, the contour of the surfaces is not a continuous one and resort is had to a method of smoothing by the use of heated implements until the normal outline is restored. The use of transparent cellophane sheets for making drawings upon, which can be viewed collectively, is recommended by van der Jagt.<sup>1</sup>

Dissections and whole embryos are often very helpful in determining the exact amount of reduction or addition necessary to restore the correct outline. There are many details involved in the process of reconstruction which cannot be given here, but for which information may be secured by consulting such works as Minot's laboratory manual.<sup>2</sup>

<sup>1</sup> *Science*, 74: 601, (July 3) 1931.

<sup>2</sup> Minot, C. S. *Laboratory Text Book of Embryology*. Ed. 2, Phila., 1910.

## CHAPTER VII

### HISTOLOGICAL METHODS

METHODS OF STUDYING RED BLOOD CELLS 287. METHODS FOR STUDY OF LEUCOCYTES 324. HISTOLOGICAL TECHNIQUE FOR STUDY OF BONE 344. HISTOLOGICAL TECHNIQUE FOR DENTAL TISSUES 353. METHODS FOR INTERCELLULAR SUBSTANCES OF CONNECTIVE TISSUES 402. METHODS FOR PREPARING MUSCLE AND ELECTRIC ORGAN TISSUES 420. NEUROLOGICAL TECHNIQUE 437. SILVER METHODS FOR BOUTONS TERMINAUX AND NEUROFIBRILS 481. NEUROGLIA AND MICROGLIA (THE METALLIC METHODS) 489.

### METHODS OF STUDYING RED BLOOD CELLS

RAPHAEL ISAACS

Counting red blood cells 287. Counting white blood cells 292. Counting blood platelets 293. Estimating hemoglobin percentage 295. Measure of hemoglobin percentage by means of oxygen combining power of blood 299. Color index 302. Determining volume of blood cells 303. Obtaining volume index 304. Mean corpuscular volume 304. Mean corpuscular hemoglobin 305. Mean corpuscular hemoglobin concentration 305. Saturation index 305. Washing red blood cells 305. Determination of specific gravity of blood 306. Determining resistance of red blood cells to hypotonic salt solutions 307. Determining resistance of red blood cells to heat 308. Determining sedimentation rate of red blood cells (suspension-stability) 309. Measuring red blood cells 311. Studying hemagglutination and hemolysis of red blood cells 314. Studying sickling of red blood cells 318. Demonstrating special features or structures in red blood cells 319. Blood platelet diluting solutions 322.

#### I. Methods of Counting Red Blood Cells

1. **Apparatus and Materials.** *Solution*, Hayem's, Toisson's or Gower's, freshly filtered (p. 323).

*Alcohol*, 70 per cent, for washing the skin.

*Blood Lancet.* A pen point may be used or a Hagedorn needle. There are special types of blood lancets on the market, some with guards to measure the depth to which the needle penetrates the skin, and others, automatic, with a point released by a spring.

*Red Blood-counting Pipette.* It is well to use one with a Bureau of Standards certificate or one which has been checked or calibrated, so that its accuracy is known. The best type of blood-counting pipette is one with a blunt tip as this is less likely to break. There are some special types of blood-counting pipettes made to overcome certain defects of the



ones commonly used. In the Trenner diluting pipette the inner end of the measuring capillary tube terminates slightly above the lower level of the mixing chamber and at right angles to its floor. Blood drawn into

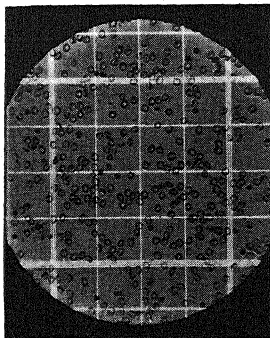


FIG. 1. Appearance of bright line hemocytometer slide.

this tube runs by capillary action to the end of the tube which is, therefore, automatic in this respect. This tube is graduated differently from the original Thoma diluting pipette in that the total volume of the capillary tube is  $\frac{1}{200}$  of the content of the bulb of the red counting pipette (for the white counting pipette the capillary tube volume is  $\frac{1}{20}$  of the volume of the mixing chamber). This pipette eliminates one error in measuring the blood for dilution. The Piney pipette<sup>1</sup> is more elaborate and is useful for very exact measurements. The Shaweker pipette has the dilutions engraved on the capillary stem instead of the figures 0.5 or 1.0 as in the Thoma pipette.

**Counting Chambers.** There are several types. The Bürker<sup>2</sup> type is now becoming more popular and gradually replacing the older type with a round moat and round center table. The newer counting chambers are made of one piece of glass and should be chosen in preference to those in which the ruled surface or the supporting surfaces are cemented to the glass slip. There are numerous types of rulings. Many of them are of historical and evolutionary interest. The simplest and most practical is the Neubauer ruling in the improved form.<sup>3</sup> The directions given here will enable one to use any type of ruling. The Spencer "bright-line" counting chamber has a metallic film incorporated in the surface of the slide on which the rulings appear as white lines against a dark background (Fig. 1).

**2. Technique.** The blood may be taken from the ball of the finger

<sup>1</sup> Piney, A. *Lancet*, 1: 906, 1924.

<sup>2</sup> Bürker, K. *Arch. f. d. ges. Physiol.*, 118: 460, 1907.

<sup>3</sup> Neubauer, E. *J. Lab. & Clin. Med.*, 10: 56, 1924. *J. A. M. A.*, 84: 947, 1925.

or the lobe of the ear. The lobe of the ear is less sensitive and the patient cannot see the manipulation. In a dog the blood can be taken from a razor cut on the margin of the ear; in a rabbit a slight cut over one of the ear veins will give sufficient blood; in a mouse the tip of the tail may be cut off and in a rat a slight cut in the tail may be used. In the guinea pig, blood may be obtained from a cut in the ear.

The place may be rubbed lightly with 70 per cent alcohol and the alcohol allowed to evaporate completely. The blood lancet should be sterile and free from old clots of blood, as these tend to make the instrument appear dull. If the instrument is chisel shaped and has a wide sharp point, a free flow of blood is obtained with the least possible manipulation.

The bottle of diluting solution should be open and easily available so that no time will be lost. The mouth piece of the pipette is held in the mouth and the lancet is plunged into the skin. The blood must flow freely. The first drop of blood is usually wiped away and with the least possible pressure a second drop is allowed to form. When a drop about 3 mm. in diameter has accumulated, the tip of the pipette, which must be chemically clean and absolutely dry, should be touched to it and the blood drawn up to the mark 0.5. If the blood is very dilute, as in a patient with profound anemia, the blood may be drawn up to the 1.0 mark. This will mean that after the final mixing it will be diluted 1 to 100 instead of 1 to 200. This must be taken into account in making the final calculation. The pipette should be held at about 45° with the point downward. Great care should be used to have the blood reach the exact line. Excess blood is wiped from the tip of the pipette and with the pressure maintained by holding the cheeks steady, the tip of the pipette is put into the diluting solution. By suction the solution is drawn up and the pipette is snapped with the finger once or twice to release the glass bead which sometimes adheres to the wall and retains an air bubble beneath it. The diluted fluid is drawn up to the mark 101, the pipette is withdrawn from the solution and the finger is immediately placed over the tip. The mouth piece is then removed from the mouth and the pipette is shaken for at least three minutes. The pipette should be shaken in such a way that all the blood will be thoroughly mixed.

A simple revolving in one direction or shaking in the longitudinal axis is usually not sufficient to include the blood which is held in the capillary ends of the diluting chamber, and motion should preferably be in several directions. Automatic shakers have been described. The suspension should be absolutely uniform with no visible clots. If the process is delayed at any stage or the manipulation is not rapidly performed, the blood may clot. The hemacytometer slide should be ready

so that the drop of diluted blood can be put under the cover glass. The cover glass and slide must be grease-free. As with the pipettes, the slides and covers should be of known accuracy, preferably with a U. S. Bureau of Standards certificate. These are best cleaned with a little alcohol, acetone or ether. If the slide is of the old type and has balsam underneath the ruled surfaces, these solvents can be used only on the cover glass. There are two kinds of hemacytometer slides in use, those with a round moat and round table which are filled before the cover glass is put on, and those with a rectangular table which are filled after the cover glass is put on. The latter type of slide is of two kinds, one with smooth glass ridges to support the cover glass and one with ground glass ridges. When a smooth glass type is used the cover glass is placed on the ridges and slight pressure exerted until Newton's colored rings (rainbow effect) can be seen where the glass surfaces come in contact. With the ground glass type the cover glass is merely put into place. Some workers place a microscopic drop of water on the ground glass surface to hold the cover glass in place. This should never be large enough to spread over the entire surface. When a cover glass which has been checked by the Bureau of Standards is used, the mark etched in the glass should be on the upper surface. Great care should be used not to disturb the cover glasses after they are once in position. Some types of hemacytometer slides are provided with clips to hold the cover glass in place. The pipette with the diluted blood is well shaken and if it has been allowed to stand any length of time, it should be shaken at least three minutes before it is used. After the blood is thoroughly mixed, one or two drops of solution are blown out to expel the portion which has been in the capillary part of the pipette. The mouth piece is held in the mouth and a drop of blood is blown underneath the cover glass until the space above the table is filled. The drop must be put under the cover glass with one sweep and must not be allowed to proceed in little waves, as the distribution of cells will then be uneven. If bubbles form underneath the cover glass, it indicates that the glassware was not grease-free and the manipulations must be repeated after cleaning the slide and the cover glass. Then with slight suction any excess of fluid is quickly removed so that the side moats are not flooded. With the older type, round moat slides, a drop, the size of which is determined by trial, is put on the top of the table and the cover glass is placed over this. This second type has the advantage that with it evaporation is prevented, but the rectangular type is more easily filled.

The cells are allowed to settle for at least two minutes and then are counted. The low power objective (16 mm.) and No. 10 ocular are used for counting. If one is not familiar with the appearance of the cells he

may use the 4 mm. objective (high-dry). The slide is moved until the smallest squares are in the field. Some slides are provided with a deeply ruled groove which points to the fine rulings used in counting the cells. This is of aid to some in finding the area to be counted. Great care must be used to have the slide absolutely level while the cells are settling and the slide must never be jarred or the cover glass moved after the blood suspension has been placed on the slide.

In counting the cells each worker should adopt some system with reference to the cells falling on lines so that no duplication will be made in the counting. Probably the simplest method is to include all cells falling on lines above and to the left of a square in the total count for that square, and to reject all cells falling on the line to the right and at the bottom of the square, as these will be included when the other squares are counted. Squares may be counted in blocks of 25 ( $5 \times 5$ ) or in blocks of 16 ( $4 \times 4$ ) and blocks may be selected running across or diagonally down the field. In some types of ruling, the blocks are separated, for convenience in counting, by double or triple ruled lines on the edges of the blocks. In others, a single line is ruled through the middle of the set of small squares which form the border of the blocks. In the former type of ruling, the double or triple ruled lines should be regarded as a single line in allocating the corpuscles which touch them. In the second type of ruling the line does not form a boundary in itself, and therefore does not enter as a factor when the cells on the edge are counted.

Automatic tally counters are sometimes of help in enumerating the cells.

Each of the smallest squares in the center of the ruled area measures  $\frac{1}{400}$  sq. mm. in area and is  $\frac{1}{10}$  mm. deep. Under ideal conditions the cells which have sedimented in 400 of these squares should be counted, representing a volume of 1 sq. mm. in area and  $\frac{1}{10}$  mm. in depth. If the cells in any smaller number of squares are counted, calculation must be made to make up the total; thus if the cells of 100 small squares are counted the number obtained will have to be multiplied by 4 to obtain the number in a square millimeter area. The greater the number of squares counted, the less the inaccuracy. In a specimen of normal blood with an average of 5,000,000 cells per cubic millimeter, 100 of the smallest squares will contain 625 cells, if the dilution is 1 to 200. Care should be taken to exclude the white blood corpuscles in counting the red cells. The former can be recognized easily by their difference in refractivity. Occasionally a few are counted with the red cells, producing a small error in the final calculation.

An example of a blood count is as follows:

Number of red cells in 100 small squares = 625.

Since there are 400 squares in 1 sq. mm., this number must be multiplied by 4 to obtain the number in a square millimeter. As the column of fluid is  $\frac{1}{10}$  mm. deep, to obtain the number in a cubic millimeter this number must be multiplied by 10. Since the dilution was  $\frac{1}{2}$  unit of blood diluted to 101 units, the suspension of corpuscles was  $\frac{1}{200}$  the concentration of the original blood. Therefore, the number of cells in  $\frac{1}{10}$  cu. mm. of the diluted suspension multiplied by 200 will give the number of cells per cubic millimeter of blood.

$625 \times 4 \times 10 \times 200 = 5,000,000$  red blood corpuscles per cubic millimeter of whole blood.

The chief sources of error are improper measurements in making the dilutions and unequal distribution of cells on the hemacytometer; the latter may be prevented to some extent by thoroughly mixing the blood suspension before it is put on the hemacytometer slide, by allowing the blood to rush under the cover glass with one sweep instead of little waves, by using great care to prevent jarring of the slide or movement of the cover glass, and by having the cover glass and slide absolutely grease-free. An experienced operator usually must count on an average error of  $\pm 5$  per cent in his red cell counts, which would mean that counts which vary by 250,000 when the absolute count is around 5,000,000 can be considered identical. When the double type of hemacytometer is used, the count can easily be made in duplicate. If a pipette with the diluted blood has stood for some time and is reshaken, the counts are not quite as accurate as when the freshly made suspension is used.

The pipette should be cleaned at once, using water to wash out the blood, then alcohol and finally ether. The alcohol and ether should be sucked through. A hand bulb or a suction apparatus may be used. It is well to have a horse hair as part of the equipment to clean out particles which become adherent to the inside of the tube. If the blood coagulates in the pipette, it should be cleaned out at once. If particles adhere to the sides of the tube they may be digested with an acid solution of pepsin. The slide and cover glass should be washed with water and the ruled area should be carefully dried with soft lens paper.

## II. Method of Counting White Blood Cells

1. **Apparatus and Materials.** The method is similar to that used in making a red blood cell count, but as a diluting solution fresh 1 per cent acetic acid is used. The pipette is calibrated so that a dilution of 1 to 10 or 1 to 20 may be obtained.

2. **Technique.** The blood is collected and drawn into the white blood cell counting pipette to the 0.5 mark for ordinary blood and to

the 1.0 mark when the white count is very low. The diluting solution is then drawn in until the mixture reaches the 11.0 mark, using the same technique as with the red blood cells (q. v.). The same precautions are used in mixing and in placing the mixture on the slide. The cells in each of the four corners of the ruled area are counted. With the ordinary ruling, 1 sq. mm. just fits into the low power field (16 mm. objective, No. 10 ocular). With the usual rulings this space is divided into sixteen squares. The cells in a minimum of 4 sq. mm. should be counted for each enumeration. Those cells falling on the lines which form the left border and the upper border of the field should be included with the cells in that area. Those falling on the lines which form the right border and the lower border should be included with the other squares.

The calculations are as follows:

Number of cells in each square millimeter area multiplied by 10 gives the number of cells in a cubic millimeter; multiplied by the dilution 20, when the blood is drawn to the 0.5 mark and by 10 when the blood is drawn to the 1.0 mark, gives the number of cells per cubic millimeter of the undiluted blood.

Example:

Number of cells per square millimeter, 45.

Dilution 20 times.

$45 \times 10 \times 20 = 9000$  white blood cells per cubic millimeter of whole blood.

3. **Citrate Method (Osgood).**<sup>4</sup> Blood is drawn from a vein into a dry 10 c.c. syringe and discharged into a test tube containing 2 mg. of dry potassium oxalate for each cubic centimeter of blood. Mix thoroughly. This blood may be used within one hour for platelet counts or a differential smear; within three hours for red-blood-cell volume, peroxidase test, fragility test or sedimentation rate; within four hours for the icterus index and Van den Bergh test; and twenty-four hours for the hemoglobin estimation, red-blood-cell and leucocyte count. Extreme care must be used to prevent evaporation (the test tube must be corked tightly) and to insure thorough re-mixing after being allowed to stand.

### III. Methods of Counting Blood Platelets

1. **Methods of Ottenberg and Rosenthal, Wright and Kinnicutt, Rees and Ecker, and Kristenson.**<sup>5</sup> Using the technique described

<sup>4</sup> Osgood, E. E., Haskins, H. D., and Trotman, F. E. *J. Lab. & Clin. Med.*, vol. 16: 1930-31.

<sup>5</sup> Ottenberg, R., and Rosenthal, N. *J. A. M. A.*, 69: 999, 1917.

Wright, J. H., and Kinnicutt, R. *J. A. M. A.*, 56: 1457, 1911.

Rees, H. M., and Ecker, E. E. *J. A. M. A.*, 80: 621, 1923.

Kristenson, A. *Acta Med. Scandinav.*, 57: 301, 1922.

under "Counting Red Blood Cells" (p. 287) and with all the glassware chemically clean, the blood is drawn from a freely flowing drop to the 1.0 mark in a red blood counting pipette and as a diluting solution the sodium citrate solution of Ottenberg and Rosenthal, or the solution of Wright and Kinnicutt or Rees and Ecker or of Kristenson may be used. The diluting solution (freshly filtered) and the blood are drawn up to the 101 mark. The blood is thoroughly mixed in the pipette and one or two drops are blown out and discarded. A drop of the diluted mixture is then allowed to run underneath the cover glass of a hemacytometer slide. This is covered with a small glass or bell jar to prevent evaporation and allowed to stand for ten minutes. If desired, the preparations (except those with the Wright and Kinnicutt and Kristenson solutions, which hemolyze the red blood cells) may be used during this time to make a red blood cell count. The platelets are counted under the high dry objective. The normal counts with these methods vary from 200,000 to 400,000. The calculations are the same as those for a red blood cell count (q. v.) the dilution being 100 times instead of 200.

Example:

Number of platelets in 1 sq. mm. area (400 of the small squares)  $\frac{1}{10}$  mm. deep, 250

In 1 cu. mm.  $10 \times 250 = 2500$

Dilution 100 times

$2500 \times 100 = 250,000$  per cubic millimeter of blood.

2. **Method of Buckman and Hallisey.**<sup>6</sup> Venous blood is drawn into a paraffined tube. The blood is then drawn into a red blood counting pipette to the 0.5 mark and the special diluting solution of Buckman and Hallisey is drawn in until the mixture reaches the 101 mark. The pipette is shaken for three minutes and after blowing out and discarding a few drops, some of the suspension is put under the cover glass of a hemacytometer slide. After allowing three minutes for the cells to settle, a red blood cell count is made in the usual way. (See p. 287, Method of Counting Red Blood Cells.) In five minutes a white blood cell count may be made from the preparation. After twenty minutes the platelets are counted, using the high dry objective. Cells in 4 square millimeters are counted. The calculations are as given under Method of Counting Red Blood Cells (p. 287). With this method counts may be made as long as four hours after the specimen is taken. The normal counts vary from 246,000 to 328,000 platelets per cubic millimeter, with an average of 284,000 per cubic millimeter. The platelets appear as discrete, uniformly distributed, pale blue, oval bodies about  $\frac{1}{6}$  to  $\frac{1}{3}$  the

<sup>6</sup>Buckman, T. E., and Hallisey, J. E. *J. A. M. A.*, 76: 427, 1921.

size of normal red blood cells. The cytoplasm of the cells appears finely granular and the periphery of the platelets slightly irregular.

3. **Method of Pratt.**<sup>7</sup> A few cubic centimeters of the special diluting solution of Pratt are placed in an absolutely grease-free watch glass. A drop of the fluid is taken up in a sterilized platinum loop (3 mm. in diameter) and is brought into contact with a freshly flowing drop of blood from a puncture wound in the ear. There should be three or more parts of fluid to each part of blood. The mixture is placed on a grease-free slide and covered with a thin cover glass. If desired the dilution may be made on the slide. The mixture should be allowed to spread so that the red blood cells are well separated. It is best to make two preparations. The red blood cell count is made from another drop of blood in the usual way, using the standard methods for this purpose. (See p. 287, Method of Counting Red Blood Cells.) The ratio of the number of blood platelets to the number of red blood cells is calculated from an enumeration of both elements in the fresh preparation. The oil immersion lens is used. A diaphragm with square opening, placed in the ocular, or an Ehrlich eyepiece may help in making the count. From 250 to 500 red blood cells should be counted in two preparations. The normal platelet count with this method varies from 226,000 to 725,000, with an average of 469,000 per cubic millimeter of blood.

4. **Direct Method.** Brilliant cresyl-blue solution is dried on cover glasses and blood films are made as in the method for staining red blood cells for reticulum (p. 319). The platelets noted in counting from 1000 to 5000 red blood cells are enumerated. Calculation: Number of platelets per 1000 red blood cells times the number of thousands of red blood cells per cubic millimeter found on making a red-blood-cell count from blood obtained at the same time that the film was made. At present this appears to be the simplest and most accurate of the methods for enumerating blood platelets. The normal is from 250,000 to 500,000 per cubic millimeter.

#### IV. Methods of Estimating Hemoglobin Percentage

1. **Sahli Method.**<sup>8</sup> Into the graduated tube supplied with the Sahli apparatus,  $\frac{1}{10}$  normal hydrochloric acid is placed until it reaches the mark 10. The blood is then drawn into the special hemoglobinometer pipette until the mark 20 cu. mm. is reached. A fairly large-sized drop of blood is needed. The tube is held vertically and the hemoglobinometer

<sup>7</sup> Pratt, J. H. *J. A. M. A.*, 45: 1999, 1905.

<sup>8</sup> Sahli, H. *Diagnostic Methods*. Edited by N. B. Potter, Phila., 1918, p. 749.



pipette is lowered into the tube until its tip is just beneath the surface of the hydrochloric acid. The blood is blown slowly into the solution until the pipette is practically empty. Then the hydrochloric acid is sucked up and gently blown out several times until all the blood is washed out. In this way bubbles are avoided and no blood is lost. At the end of exactly sixty seconds water is added and gently mixed with the blood and hydrochloric acid solution, using a fine, bead-tipped glass rod. Water is added drop by drop and the solutions mixed, and the color is compared with the standard tubes which accompany the apparatus. When enough water has been added to make the color of the blood mixture and that of the standard similar, the height of the column of fluid is read and the number is the percentage of the hemoglobin. The solid glass standard tubes have the advantage over liquid hematin hydrochloride suspensions inasmuch as the color of the latter tends to fade. The Sahli pipette is cleaned with water and then with alcohol and ether. In this method 100 per cent is equivalent to 14.0 or to 17.3 gm. of oxyhemoglobin per 100 c.c. of blood, depending on the standard used.

**2. Tallqvist Method.** A drop of blood is taken up on the special blotting paper supplied with the Tallqvist books, which are on the market. After the drop of blood has just dried and the gloss is no longer visible, the blotting paper is bent so that there is a white sheet behind the drop of blood. With the paper in this position the blood stain is held opposite one of the holes in the color chart supplied with the book. The paper is then moved from hole to hole until the colors are matched. The reading then gives the percentage of hemoglobin. In this method, 100 per cent is equivalent to 15.8 gm. per 100 c.c. of blood.

**3. Dare Method.** The blood is taken between two plates of the blood "pipette" which is supplied with the colorimeter. This is quickly transferred to the instrument so that the glass plate is toward the observer. A yellow light (either a candle or electric light) is used and the color is compared with the standard glass plate and the latter is moved until the colors, as seen through the eye piece, appear identical. The reading is then made on the wheel on the side of the machine, and this is taken as the percentage of hemoglobin. In this method, 100 per cent is equivalent to 13.77 or 17.2 gm. per 100 c.c., depending on the standard used.

**4. Newcomer Method.<sup>9</sup>** A special hemoglobinometer, a modified Duboscq colorimeter, is used. The instrument has two cups, one filled with plain water and the other with the blood preparation. The latter is made by diluting 10 cu. mm. of blood drawn in a special pipette supplied with the apparatus, to 50 volumes, with 5 c.c. of 1 per cent hydrochloric

<sup>9</sup> Newcomer, H. S. *J. Biol. Chem.*, 55: 569, 1923.

acid. With this dilution readings down to 40 per cent hemoglobin may be obtained, but for lower values 20 cu. mm. of blood must be drawn into the pipette. The standard used for comparison is a piece of colored glass superimposed over the cup containing the plain water. The instrument is calibrated to read in percentage of hemoglobin when the color of the hematin-hydrochloric suspension is practically at its maximum depth. On this scale 100 per cent corresponds to 16.92 gm. of hemoglobin per 100 c.c. of whole blood. After the solution has stood for one-half hour, the readings are correct to within approximately 1 per cent. Readings made before this time must be corrected by the use of figures given in a table which accompanies the instrument. If the standard glass slip is not exactly 1 mm. in thickness, correction must be made for this from a table supplied with the instrument.

**5. Hemoglobin Determination. Osgood-Haskins Method.**<sup>10</sup> Oxalated venous blood or blood from the tip of the ear or finger may be used. One cubic centimeter is measured into a 100 c.c. volumetric flask containing 40 c.c. distilled water, or a proportional fraction of each may be used (e.g., 0.05 c.c. in 2.45 c.c. water). After laking, about 50 c.c. (or corresponding fraction of N/5 hydrochloric acid is added, and the mixture is diluted to the proper volume. Foam may be cut with a drop of ethyl alcohol. The solutions are mixed thoroughly, heated in a bath at 55-60° for at least seven minutes and then allowed to cool. Instead of heating the solution, it may be allowed to stand for twenty-four hours. The solutions are compared in a colorimeter against a known acid-hematin standard or against a standard ferric sulphate-chromium sulphate solution. Correction for the temperature of the standard solution is made from published tables if the inorganic standard has been used or for the reading at which the standard solution was calibrated, if the acid-hematin standard is used. In this method, 13.8 gm. per 100 c.c. equals 100 per cent.

**6. Haden-Hausser Method.**<sup>11</sup> Two models of the special hemoglobinometer are available, a laboratory and a clinical form. The principle is the same in both, but the following directions apply specifically to the 1935 clinical model.

The comparator slide and cover glass are thoroughly cleaned with a strong (saturated) solution of sodium bicarbonate, followed by rinsing in running water and drying with a soft cloth. At all subsequent stages, care must be used to avoid getting grease on the glass surface. The

<sup>10</sup> Haskins, H. D., and Osgood, E. E. *Northwest Med.*, 25: 500, 1926.

Osgood, E. E., Haskins, H. D., and Trotman, F. E. *J. Lab. & Clin. Med.*, vol. 16, 1930-31.

<sup>11</sup> Haden, R. L. *J. Lab. & Clin. Med.*, 16: 68, 1930-31; 18: 1062, 1932-33; vol. 20: 1934-35.

cover glass is placed in position with its beveled side in contact with the comparator slide. The notch should be at the observer's right, so as to coincide with the transverse depression in the comparator slide. The

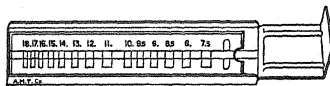


FIG. 2. Comparator slide with cover glass in position.

lower and left edges of the cover should be in contact with the corresponding walls of the metal frame encasing the slide.

The blood is drawn from a freely flowing drop into a white blood

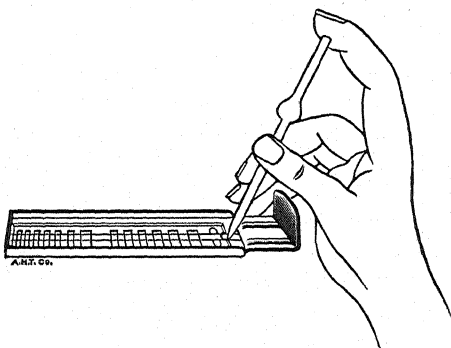


FIG. 3. Method of filling comparator slide.

cell counting pipette, up to the "0.5" mark. Special pipettes are available. Hydrochloric acid (N/10) is drawn into the pipette until the mixture reaches the "2" mark. The pipette is then thoroughly shaken, and

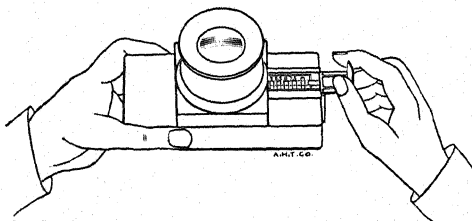


FIG. 4. Method of inserting filled comparator slide in the instrument.

the contents of the capillary stem of the pipette are blown out. After thirty minutes (room temperature) the contents are allowed to flow under the cover glass by capillarity, filling the chamber.<sup>12</sup> By trial, the

<sup>12</sup> If a shorter time is desired, corrections must be made as follows: ten minutes, add 4 per cent to the reading in grams; fifteen minutes, add 3 per cent; twenty minutes, add 2 per cent.

colors are matched through the eyepiece. In the clinical model, daylight or Mazda electric light is used; in the laboratory model, a light accompanies the machine. The amount of hemoglobin is read in *grams per 100 c.c.*, interpolations being made between the printed figures when necessary. Haden's standard for normal with this method was 15.4 gm. per 100 c.c. blood corresponding to approximately 5,000,000 red blood cells per cubic millimeter in normal individuals.

## V. The Measure of Hemoglobin Percentage by Means of the Oxygen Combining Power of the Blood

1. Method of Van Slyke and Stadie, Modified by Lundsgaard and Möller.<sup>13</sup> A Van Slyke gas apparatus is cleaned and filled with mercury. Distilled water, 6 c.c., is placed in the cup and 2 to 3 drops octyl alcohol and 0.3 c.c. of a 1 per cent solution of saponin in water are added. The upper stop-cock is opened and the solution drawn down into the apparatus so that the upper level of the solution is at the upper stop-cock. This stop-cock is then closed and the mercury lowered until its level is at the lower stop-cock. This will create a reduced pressure over the water-alcohol-saponin solution and some of the contained gases will be extracted. The machine should be shaken to facilitate this process. The lower stop-cock is so turned that when the mercury is lowered still further the solution will run down into the lower bulb where it is trapped. By turning the lower stop-cock 180° the mercury is allowed to run up into the mixing bulb and capillary pipette. The air which is gathered above this is released through the upper stop-cock. The mercury is then lowered so that the capillary stem and mixing chamber are completely empty and by turning the lower stop-cock 180° the water-alcohol-saponin solution is again allowed to run into the mixing bulb. The apparatus is thoroughly shaken. The solution is then run back into the lower bulb and the air which has been released from the liquid again trapped over the mercury and expelled through the upper stop-cock by allowing the mercury to fill the mixing bulb and capillary pipette. This process must be repeated several times until no more air can be withdrawn from the fluid. The fluid is then drawn into and trapped in the lower bulb, and the mixing bulb and capillary pipette are filled with mercury.

Blood, 5 to 10 c.c., is withdrawn from a vein and mixed with 0.1 c.c. of a 20 per cent solution of sodium citrate or 0.1 c.c. of a 2 per cent solution of heparin in water. These will prevent coagulation. Blood, 3 to 5

<sup>13</sup> Van Slyke, D. D. *J. Biol. Chem.*, 33: 127, 1918.

Van Slyke, D. D., and Stadie, W. C. *J. Biol. Chem.*, 49: 1, 1921.

Lundsgaard, C., and Möller, E. *J. Biol. Chem.*, 52: 377, 1922.

c.c., is placed in a separatory funnel and the funnel is rotated so that the blood forms a thin film on the sides. The upper stop-cock is removed occasionally to insure saturation of the blood with oxygen. The blood will usually be saturated after about two to three minutes' exposure in this way. Of the oxygenated blood, 2 c.c. are placed in the upper cup of the gas apparatus and 2 or 3 drops of octyl alcohol placed over it. The upper stop-cock is slowly opened and the mercury level lowered until the blood is entirely within the graduated capillary stem. The mercury can be lowered until some of the supernatant octyl alcohol also enters. Several drops of mercury are put in the cup and allowed to be sucked into the capillary of the upper stop-cock. The latter is tightly closed and the mercury lowered until its upper level is at that of the lower stop-cock. The stop-cock is then turned  $180^\circ$  so that the contained water-octyl-alcohol-saponin solution runs into the mixing chamber and mixes with the blood. The two are shaken together and after about one-half to one minute the blood is completely laked. The mercury is then allowed to run in through the lower stop-cock until the laked blood solution again fills the graduated capillary pipette. There will be a little gas over this solution. Of a 20 per cent air-free potassium ferricyanid solution (which has been boiled and preserved under liquid petrolatum) 0.1 c.c. is carefully placed in the cup and by turning the upper stop-cock is allowed to run into the blood mixture without loss of the imprisoned air. The upper stop-cock is filled with mercury and the mercury level is lowered until it reaches that of the lower stop-cock.

The blood mixture is thoroughly shaken for at least one minute. At this stage the oxygen is released from the hemoglobin. The blood mixture is then drawn into the lower bulb and the stop-cock turned  $180^\circ$  and the mercury allowed to fill the mixing bulb and the capillary pipette. The mercury bulb is held against the capillary pipette so that the level of the mercury in the bulb and the level of the mercury in the pipette are the same. A little fluid is usually imprisoned over the mercury. The top of this column is used as the meniscus in reading the height of the column of gas which partly fills the graduated capillary tube. This reading is noted. The mercury is then lowered until its level is that of the lower stop-cock. The blood mixture is then allowed to run into the mixing chamber and the shaking process repeated. It is then again imprisoned in the lower bulb and mercury is allowed to fill the mixing bulb and the graduated capillary stem. After leveling the mercury bulb with the mercury in the capillary stem the reading of the meniscus above the fluid column above the mercury is again taken. The whole process should be repeated several times until this reading becomes constant. The amount of gas imprisoned represents the oxygen liberated from

the 2 c.c. of blood at the temperature and barometric pressure of the room. This gas contains the air which was mechanically dissolved by the blood as well as the oxygen which was held by the hemoglobin. From the table (Table II) the amount of air physically dissolved by the blood may be obtained and this should be subtracted from the observed reading. To obtain the hemoglobin percentage the corrected reading must then be multiplied by the factor given in Table II. With this method, 100 per cent is equivalent to 15.6 gm. of oxyhemoglobin per 100 c.c. of blood.

The apparatus may be cleaned by washing repeatedly with 5 per cent ammonia water, followed by distilled water.

TABLE I  
FACTORS FOR CORRECTION FOR BAROMETRIC PRESSURE

Barometer	$\frac{B}{760}$	Barometer	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.010
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

2. **Manometric Method of Van Slyke and Neill.**<sup>14</sup> The oxygen combining power of hemoglobin is determined by a manometric method, after release of oxygen from hemolysed blood (saponin) with potassium ferricyanide. For the details of manipulation and many sources of technical error, together with the tables, it is advisable to consult the original article. A manometric method based on the carbon-monoxide combining power of hemoglobin may also be used.<sup>15</sup>

3. **Hemoglobin: Photo-electrometer Method.**<sup>16</sup> The amount of hemoglobin may be calculated from the readings on the ammeter of a

<sup>14</sup> Van Slyke, D. D., and Neill, J. M. *J. Biol. Chem.*, 61: 554, 1924.

<sup>15</sup> Van Slyke, D. D., and Hiller, A. *J. Biol. Chem.*, 78: 807, 1928.

<sup>16</sup> Kennedy, R. P. *Am. J. Physiol.*, 79: 346, 1927.

Sanford, A. H., and Sheard, C. *J. Lab. & Clin. Med.*, 15: 483, 1929-30.

Sanford, A. H., Sheard, C., and Osterberg, A. E. *Am. J. Clin. Path.*, 3: 405,

TABLE II  
FACTORS FOR CALCULATING RESULTS FROM ANALYSIS OF 2 C.C. OF BLOOD  
SATURATED WITH AIR

Room Temperature ° C.	Gas Physically Dissolved by 2 c.c. of Blood. Subtract This Figure from the Observed Reading to Obtain Volume of Oxygen Liberated from the Hemoglobin c.c.	Factor by Which the Corrected Gas Volume is Multiplied to Give Percentage of Hemoglo- bin. (20.9 Per Cent Oxygen Corresponds to 100 Per Cent Hemoglobin in This Table) Per Cent
15	0.037	$224 \times \frac{B^*}{760}$
16	0.036	$223 \times \frac{B^*}{760}$
17	0.036	$222 \times \frac{B^*}{760}$
18	0.035	$221 \times \frac{B^*}{760}$
19	0.035	$220 \times \frac{B^*}{760}$
20	0.034	$218 \times \frac{B^*}{760}$
21	0.033	$217 \times \frac{B^*}{760}$
22	0.033	$216 \times \frac{B^*}{760}$
23	0.032	$215 \times \frac{B^*}{760}$
24	0.032	$214 \times \frac{B^*}{760}$
25	0.031	$213 \times \frac{B^*}{760}$
26	0.030	$212 \times \frac{B^*}{760}$
27	0.030	$211 \times \frac{B^*}{760}$
28	0.029	$209 \times \frac{B^*}{760}$
29	0.029	$208 \times \frac{B^*}{760}$
30	0.028	$207 \times \frac{B^*}{760}$

\* B = Barometric reading.

photo-electrometer. Tables and curves have been published to simplify the calculations.

## VI. Color Index

The color index of the blood is the quotient obtained by dividing the percentage of hemoglobin (obtained from the readings of one of the

hemoglobinometers) by the percentage of red blood cells, considering 5,000,000 per cubic millimeter as the normal (100 per cent). Since the red blood cell count of a normal person may vary almost 2,000,000 per cu. mm. during the course of the day<sup>17</sup> and since the hemoglobin may vary from 15 per cent to 20 per cent during the same period, and considering also that the percentage of hemoglobin and number of red blood corpuscles may be slightly lower in women than in men, the number is more or less arbitrary and to be strictly accurate must be corrected for such factors as sex, age, standard of hemoglobinometer used, diurnal variation in the hemoglobin and red blood cell count. Under ideal conditions the index would be 1; when there is a great discrepancy between the number of red blood cells and the amount of hemoglobin, the index will be below 1 when the hemoglobin *per se* is deficient, and above 1 when there is more hemoglobin in each cell than normal.

## VII. Methods of Determining the Volume of Blood Cells

1. **Macro Method.** Blood, 10 c.c., is added to a graduated centrifuge tube containing a few crystals of sodium citrate or oxalate, or 0.1 c.c. of a 2 per cent solution of heparin. The blood is mixed thoroughly and is centrifuged at 3000 revolutions per minute for thirty minutes. The volume of corpuscles can then be read and the percentage obtained by multiplying by 10.

2. **Micro Method (Van Allen).** A drop of fresh blood from a lancet cut in the skin is drawn exactly to the top of the capillary part of the special hematocrit tube of Van Allen. Sodium oxalate solution (1.3 per cent) is then drawn into the tube until the bulb is about half filled. The bottom of the tube is closed with a rubber band or preferably a special clip which can be bought with the apparatus, and the tube is centrifuged for fifteen minutes at 2700 revolutions per minute. The relative volume of corpuscles is read directly in percentage from the scale.

3. **Hematocrit (Wintrobe).**<sup>18</sup> Venous blood is added to a test tube containing 10 mg. of dry neutral potassium oxalate<sup>19</sup> per 5 c.c. of blood. After thorough mixing, blood is added to a special Wintrobe centrifuge tube to the "10" mark, using a filling pipette. This should be lowered

<sup>17</sup> Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A. *Johns Hopkins Hosp. Bull.*, 37: 14, 1925.

<sup>18</sup> Wintrobe, M. M. *J. Lab. & Clin. Med.*, 15: 287, 1929-30. *Am. J. M. Sc.*, 185: 58, 1933.

<sup>19</sup> This proportion of potassium oxalate causes a shrinkage of 8.15 per cent. The observed volume of packed cells must be multiplied by 1.09. Instead of this, a solution of ammonium oxalate 0.6 gm., potassium oxalate 0.4 gm. in 100 c.c. of water may be made and 1 c.c. dried in a test tube in an oven. This is sufficient for 5 c.c. of blood and the shrinkage is negligible.



to the bottom of the tube and slowly withdrawn as the blood is added. The tube is stoppered to prevent evaporation, and is centrifuged for at least 15 minutes at 3000 revolutions per minute. It may be necessary to centrifuge for 30 minutes to produce adequate packing of the cells. The number opposite the upper level of the cells (in millimeters) multiplied by 100 gives the percentage volume of the corpuscles.<sup>19</sup> Normally it is about 42 per cent in women and 46 per cent in men.

A more clear cut separation of the white corpuscles and platelets from the red blood cells may be obtained if the tube is allowed to stand for about an hour before centrifuging. During this time the sedimentation rate of the corpuscles may be observed.

### VIII. Method of Obtaining Volume Index<sup>20</sup> (Capps)

The term "volume index" is used to express the quotient obtained by dividing the percentage volume of red blood cells by the percentage number of red blood cells. The percentage volume of red blood cells is obtained by centrifuging whole blood either with or without an anticoagulant (before the blood has coagulated) in a hematocrit for three minutes at about 800 to 1000 revolutions per minute. The volume of red blood cells in normal individuals is taken as 4.6 c.c. per 10 c.c. of blood. The formula for the volume index is:

$$\frac{\text{Volume of red blood cells}}{4.6} \text{ divided by } \frac{\text{Number of red blood cells per cu. mm.}}{5,000,000}$$

In normal people this number is 1.0; in pernicious anemia it is greater than unity during the relapses, and in secondary anemias, less than unity. As the average cell volume in women (4.2 c.c. per 10 c.c. blood) is less than in men and the average corpuscle number is somewhat lower, unless proper corrections are made, the index is arbitrary.

### IX. Mean Corpuscular Volume<sup>21</sup>

Formula:

$$\frac{\text{Volume of corpuscles (c.c. per 1000 c.c.)}}{\text{R.B.C. (in millions per cu. mm.)}} = \text{number of cubic microns.}$$

The volume of corpuscles is obtained by one of the hematocrit methods. If necessary, a correction is made for shrinkage caused by the anticoagulant. The percentage volume  $\times 10$  is divided by the number which represents the number of millions of red blood corpuscles ob-

<sup>20</sup> Haden, R. L. *Arch. Int. Med.*, 31: 767, 1923.

tained by counting the cells in a part of the sample of venous blood before it is centrifuged. In normal blood the average volume of a red blood cell is between 80 and 95 cubic microns.

### X. Mean Corpuscular Hemoglobin<sup>21</sup>

Formula:

$$\frac{\text{Grams of hemoglobin calculated per 1000 c.c.}}{\text{Red blood cells count, in millions per cu. mm.}} = \frac{\text{Mean corpuscular hemoglobin}}{\text{(in micro-micrograms)}}$$

Normally the mean corpuscular hemoglobin varies from 24.5 to 34.7 micro-micrograms ( $10^{-12}$  gm.), (average 28.7) in men, and 23.7 to 33.8 (average 28.6) in women.

### XI. Mean Corpuscular Hemoglobin Concentration (Proportion of Hemoglobin in Red Blood Cell)<sup>22</sup>

Formula:

$$\frac{\text{Grams of hemoglobin per 100 c.c. of blood}}{\text{Volume of corpuscles per 100 c.c. of whole blood}} = \frac{\text{Proportion of hemoglobin in cell in per cent.}}$$

In normal men, the average is about 33.7 per cent (29.9-42.6 per cent). In women the average is about 32.5 per cent (28.1-37.2 per cent).

### XII. Saturation Index (Haden<sup>23</sup>)

Formula:

$$\frac{\text{Hemoglobin per cent}}{\text{Volume per cent}} = \text{Saturation index.}$$

The hemoglobin per cent varies with age, sex and hemoglobin standard. The volume per cent is the observed volume divided by the ideal average normal volume (42 per cent in women; 46 per cent in men)  $\times 100$ .

### XIII. Method of Washing Red Blood Cells

An anticoagulant (a few crystals of sodium citrate or oxalate or 0.1 c.c. of a 2 per cent aqueous solution of heparin per 10 c.c. of blood) is added to a volume of blood and the red blood cells are sedimented by centrifugalization. The plasma is withdrawn with a pipette, and a physi-

<sup>21</sup> Wintrobe, M. M. *Am. J. M. Sc.*, 177: 513, 1929.

<sup>22</sup> Wintrobe, M. M. *J. Lab. & Clin. Med.*, 17: 899, 1931-32.

<sup>23</sup> Haden, R. L. *Arch. Int. Med.*, 21: 765, 1923.

ologic saline solution (0.85 to 0.75 per cent sodium chloride; Ringer's solution; Locke's solution; Tyrode's solution, or their various modifications) is added to make the original volume. The corpuscles are mixed with the solution and re-sedimented by centrifugalization. The salt solution can be withdrawn and the process repeated several times. Usually 3 washings are necessary to remove all traces of plasma.

#### XIV. Method for the Determination of the Specific Gravity of the Blood

1. **Method of Hammerschlag.** The simplest laboratory method for determining the specific gravity of whole blood is that of Hammerschlag.<sup>24</sup> Mixtures of chloroform (sp. gr. 1.485) and benzol (sp. gr. 0.88) are made in a wide test tube. A drop of freshly drawn blood is allowed to fall into the mixture from a pipette held just over the surface of the liquid. If the drop remains stationary when suspended in the liquid, it is assumed that its specific gravity is the same as that of the liquid. If it sinks, it is heavier and more chloroform must be added and, after mixing, the solution tested with a fresh drop of blood. If the drop remains on top it is lighter and more benzol must be added. After adding either the benzol or chloroform to change the specific gravity, the fluid must be stirred thoroughly. When the proper mixture is obtained, the specific gravity of the solution is determined by the use of a hydrometer. The specific gravity of normal whole blood varies between 1.050 and 1.062. The specific gravity of plasma or serum (1.029 and 1.023) may be determined by the same method. The determinations must be carried on as rapidly as possible as the solvents used extract lecithin and cholesterol from the corpuscles and the red blood cells become distorted and hemolyzed.

2. **Method of Reznikoff.**<sup>25</sup> The specific gravity of the *red blood cells* is best determined by the method of Reznikoff.

Mixtures of benzyl benzoate (sp. gr. 1.115 at 20°C.) and cottonseed oil (sp. gr. 0.920 at 20°C.) are placed in separate test tubes, the first containing 5 parts each and the successive tubes containing less cottonseed oil, the last tube containing pure benzyl benzoate. These are arranged in a series of small Wassermann tubes, each containing 2 c.c. of the mixture. The range of specific gravity will be from 0.017 to 1.115. A variation of 0.02 c.c. of either fluid will correspond to a change in the specific gravity of 0.002. The red cells are prepared by centrifugalizing defibrinated blood in small Wassermann tubes for fifteen minutes at 3600 revolutions per minute. The plasma is carefully withdrawn with a

<sup>24</sup> Hammerschlag, A. *Ztschr. klin. Med.*, 20: 444, 1892.

<sup>25</sup> Reznikoff, P. J. *Exper. Med.*, 38: 441, 1923.

pipette, leaving only the solidly packed red corpuscles. Sufficient blood can be obtained from a skin puncture in the ear or finger using a drop of 2 per cent heparin solution per 1 c.c. of blood as an anticoagulant. This can be centrifugalized in a small hematocrit tube and the corpuscles withdrawn with a capillary pipette. Drops of this thick sediment are placed in the various tubes and in the tube where the corpuscle mass remains suspended in the liquid, not rising to the top or falling to the bottom, the specific gravity is about that of the red corpuscles. The blood should be added to the oil mixture from a fine capillary pipette, using a rubber bulb to express the contents. A small drop of blood should be squeezed out and allowed to hang on the end of the pipette. This should then be plunged into the oil solution until it reaches about the middle of the tube. The drop can then be dislodged and it will either remain stationary, rise or sink. The specific gravity of this solution can be determined by testing a solution of similar composition with a hydrometer. The method is rapid and quite accurate for clinical purposes. The specific gravity of the normal red blood cells by this method varies from 1.092 to 1.094.

#### **XV. Method of Determining Resistance of Red Blood Cells to Hypotonic Salt Solutions**

The concentration of a solution of sodium chloride in which red blood corpuscles begin to hemolyze may be determined by the following method.

In performing this test, whole blood or washed corpuscles may be used. The blood may be drawn from a vein, but at times sufficient blood may be obtained from a lancet wound in the ear or finger. The washing process, using Ringer's solution or a similar physiologic solution, always alters the composition of the red blood cells. To wash the corpuscles, the sedimented cells, obtained by centrifugalization, are suspended in the washing solution and re-centrifugalized. This process may be repeated one or two times. The red cells are also injured during the process of centrifugalization in separating the corpuscles from the plasma. Another modification is to centrifugalize whole blood to which an anticoagulant has been added, and use the corpuscles without washing or other procedure. For comparative purposes the same method must always be used. When the whole blood is used, coagulation may be prevented by means of 0.1 c.c. of a 2 per cent aqueous solution of heparin per 10 c.c. of blood. The blood may be made into a 0.2 per cent solution of sodium citrate or oxalate by the addition of 0.2 gm. of either of these salts in powder or solution form to each 100 c.c. of blood or in this proportion.

For the salt solution, 1 gm. chemically pure, dry sodium chloride is dissolved in sufficient chemically pure, neutral distilled water to make 100 c.c. The solution should have a hydrogen-ion concentration of pH 7.0. This is the stock solution. Small, chemically clean test tubes (about  $70 \times 10$  mm.) are arranged in a rack and from two burettes salt solution or neutral distilled water is added to the tubes in varying proportions. For the preliminary test the variations from tube to tube may be made by adding 1.4 c.c. of salt solution and 0.6 c.c. of distilled water to the first tube, 1.2 c.c. of salt solution and 0.8 c.c. of the distilled water to the second tube, etc. This will give 2 c.c. of solution in each tube. The first tube will correspond to a 0.7 per cent sodium chloride solution, the second tube to a 0.6 per cent and so on. When the beginning point of hemolysis is determined, very fine gradations may be made in the strength of the salt solution between crucial points of beginning hemolysis and complete hemolysis.

A drop of the whole blood or of the corpuscle suspension is added to each tube and thorough mixture obtained by stirring with a fine glass rod, or inverting the covered tubes. The solutions are placed in an ice box and allowed to stand until complete sedimentation of the red blood corpuscles has occurred. It is best to take the readings in about twelve hours and again in twenty-four hours. Several points are noted: first the strength of the salt solution in which the first trace of hemolysis (salt solution tinged with hemoglobin) is seen. A record is made of the first tube (highest concentration of salt solution) in which hemolysis is complete. In this tube the salt solution will be deeply red and no non-hemolyzed blood corpuscles will be seen macroscopically in the bottom of the tube. In health the minimum resistance (strength of solution in which first hemolysis is evident) is 0.47 and complete hemolysis is noted in the tube in which the strength of the salt solution is 0.30.

As a control a specimen of normal blood should always be examined at the same time as the pathologic specimen.

## XVI. Method of Determining Resistance of Red Blood Cells to Heat

1. **Method of Isaacs, Brock and Minot.**<sup>26</sup> The blood is drawn from an arm vein and mixed with sufficient crystalline sodium citrate to make a 0.2 per cent solution. Blood films are then made on cover glasses on which brilliant cresyl blue has been dried. These are counterstained with Wright's stain. These preparations are used to study the condition of the blood before it is subjected to heat. Blood, 1 c.c., is placed in a small

<sup>26</sup> Isaacs, R., Brock, B., and Minot, G. R. *J. Clin. Invest.*, 1: 425, 1925.

test tube ( $70 \times 10$  mm.) and this is suspended in a water bath at a temperature ranging from  $55^{\circ}$  to  $58^{\circ}\text{C}$ . The blood of a normal individual may be used as a control. The test tube remains in the water bath for thirty minutes. It is then gently shaken to mix the blood thoroughly and blood films are made on cover glasses on which brilliant cresyl blue has been dried. In blood treated by this method, the older red cells become fragmented and hemolyzed under these conditions, whereas those showing signs of youth and a few of those which appear to be adult, remain intact. The latter are probably the younger of the adult cells. In blood in which most of the cells are young (chronic hemolytic jaundice) the bulk of the cells remain intact.

### XVII. Methods of Determining the Sedimentation Rate of Red Blood Cells (Suspension-Stability)

1. **Method of Fahraeus.**<sup>27</sup> Blood is taken from an arm vein and placed directly in test tubes 17 cm. in length and about 9 mm. in inner diameter. The tubes contain 2 c.c. of a 2 per cent sodium citrate solution. The blood is added until the contents reach the 10 c.c. mark. The ratio of citrate to blood is 1 to 4, and the height of the citrate blood mixture is about 150 mm. The tube is inverted several times to insure thorough mixture of the contents and is then allowed to stand in a vertical position. The height of the clear supernatant plasma layer is measured at the end of one hour. With this method the average is  $\frac{1}{2}$  mm. per hour. During pregnancy the rate of settling of the corpuscles is more rapid, being on the average 44.9 mm. per hour. With this method, values higher than 9 mm. per hour for men and 12 mm. per hour for non-pregnant women are considered normal.

2. **Method of Linzenmeier.**<sup>28</sup> Blood is drawn from a vein in a 1 c.c. syringe and 0.8 c.c. placed in a special tube which contains 0.2 c.c. of a 5 per cent solution of sodium citrate. The tube measures  $6\frac{1}{2}$  cm. in length and the diameter is 5 mm. It is marked in two places; mark No. 1 indicates a volume of 1 c.c. and mark No. 2 is 18 mm. lower. The tube is inverted twice to insure mixing and the time is noted. The time that it takes for the corpuscles to sink from the 1 c.c. mark to the 18 mm. mark is noted and is the time used for comparison with other bloods.

3. **Method of Zeckwer and Goodell.**<sup>29</sup> Of a 3 per cent sodium citrate solution 2 c.c. are placed in a 15 c.c. centrifuge tube graduated at 0.1 c.c. intervals. Blood from the patient's vein is put into the tube up to the

<sup>27</sup> Fahraeus, R. *Acta Med. Scandinav.*, 55: 70, 1921.

<sup>28</sup> Linzenmeier, G. *Deutsche. med. Wchnschr.*, 48: 1023, 1922.

<sup>29</sup> Zeckwer, I. T., and Goodell, H. *Am. J. M. Sc.*, 169: 209, 1925.

10 c.c. mark. The blood and the citrate are thoroughly mixed and the tube is allowed to stand in a vertical position. The time is then recorded. The height of the column of red cells is noted at the end of one hour. The height of the plasma may be obtained by subtracting the height of the red blood corpuscles from the total height of the column of liquid. If the height of the plasma is found to be slightly above or below the 10 c.c. mark, the readings for the red blood cells must be corrected proportionately. In normal individuals the rate is 8.0 to 9.8 c.c. in the first hour.

4. **Method of May.**<sup>30</sup> Venous blood is placed in a test tube 10 cm. in length and 6 mm. in diameter. The tubes stand in a special rack at the back of which is a cardboard scale with a gradation from 0 to 100. The blood is drawn from a vein into a syringe which has been washed with a 3.8 per cent solution of sodium citrate and which contains 0.4 c.c. of this solution. Blood, 2 c.c., is drawn into the syringe and mixed with the citrate solution. Of the citrated blood 1.8 c.c. is introduced into the special test tube. Care must be used to avoid the introduction of bubbles of air. The tube is allowed to stand for one hour in a vertical position when the height of the column of plasma above the red corpuscles is measured. With this method the cells for normal men are from 1 to 4 and for women 1 to 6. Figures up to 20 indicate a slight increase, up to 40 a medium increase, and a great increase if above this number. The blood should be taken from the patient while he is fasting.

5. **Finger Puncture Method of Cutler.**<sup>31</sup> Blood, 0.5 c.c., is obtained by the puncture of the palmar surface of a finger and is collected in a small test tube, the sides of which have been wet with a 3 per cent sodium citrate solution. The blood is drawn in a special pipette 2.5 mm. in internal diameter with a stem graduated in 50 mm. divisions. The capacity of the graduated portion of the pipette is less than 0.3 c.c. A Van Allen spring attachment is used for closing the bottom of the pipette after it is filled with blood. The pipette is placed upright in a sedimentation rack and the position of the upper level of the sedimentation column of red blood cells is recorded every five minutes for one hour. This may be plotted in a graphic way using the number of millimeters as the abscissas and the time in minutes as the ordinates. If many specimens of blood are taken at one time, they may be set aside for several hours if necessary until it is convenient to start observations. When observations are to be begun the tubes are inverted several times until the corpuscles are uniformly distributed once more.

<sup>30</sup> Piney, A. *Recent Advances in Hematology*, Lond., 1927, p. 261.

<sup>31</sup> Cutler, B. I. *Am. J. M. Sc.*, 178: 687, 1927.

6. **Method of Plass and Rourke.**<sup>32</sup> Blood (5 c.c.) is placed in a tube in which 0.1 c.c. of solution containing 1.5 mg. of heparin (75.0 mg. heparin per 5 c.c. distilled water) has been dried. After thorough mixing, the blood is allowed to stand for fifteen minutes (not more than three hours) to reach room temperature. It is then thoroughly mixed by tipping up and down for two to three minutes. With a fine pipette inserted to the bottom, blood is introduced into a special sedimentation tube to the mark "o." It is allowed to stand at room temperature for one hour and the length of the plasma column is recorded. The tube is then centrifuged for twenty minutes at 2500 r.p.m. and the length of the plasma column is again noted. Calculations: Divide the number representing the millimeters of clear plasma obtained after one hour by the number obtained after centrifuging and multiply by 100. This gives the per cent of the observed settling of the total possible settling in one hour. The upper limit for normal women is 65 per cent and for normal men 25 per cent.

7. **Method of Correcting Sedimentation Rate for Variations in Cell Volume Percentage of Blood.** Rourke and Ernstene.<sup>33</sup> The blood is prepared according to the method of Plass and Rourke. Readings of the clear plasma column length are recorded and plotted as long as the rate of settling is a "straight line." The number of millimeters of fall of the blood-cell meniscus during the "straight line" period, divided by the number of minutes that elapsed during this fall, gives the number of millimeters per minute. By reference to the chart (Fig. 5) the sedimentation rate can then be corrected to a hematocrit volume of 45 per cent by finding the line on which the observed number of millimeters per minute and the observed hematocrit fall and tracing it to the 45 line (Fig. 5). The normal limits of the "corrected sedimentation index" are 0.08 to 0.35 mm. per minute.

### XVIII. Methods of Measuring Red Blood Cells

1. **Ocular Micrometer.** The magnification value of the lines engraved in the ocular micrometer are determined by measuring lines, whose distance apart is known, on a stage micrometer. In the absence of the latter a hemacytometer slide may be used. A well fixed and stained blood film is placed on the stage of the microscope and the individual cells are viewed through the ocular micrometer. The slide is moved with the mechanical stage and as each cell falls under the micrometer rulings, it is measured. Usually one diameter is measured and the error caused

<sup>32</sup> Plass, E. D., and Rourke, M. D. *J. Clin. Invest.*, 5: 531, 1928.

<sup>33</sup> Rourke, M. D., and Ernstene, A. C. *J. Clin. Invest.*, 8: 545, 1930.



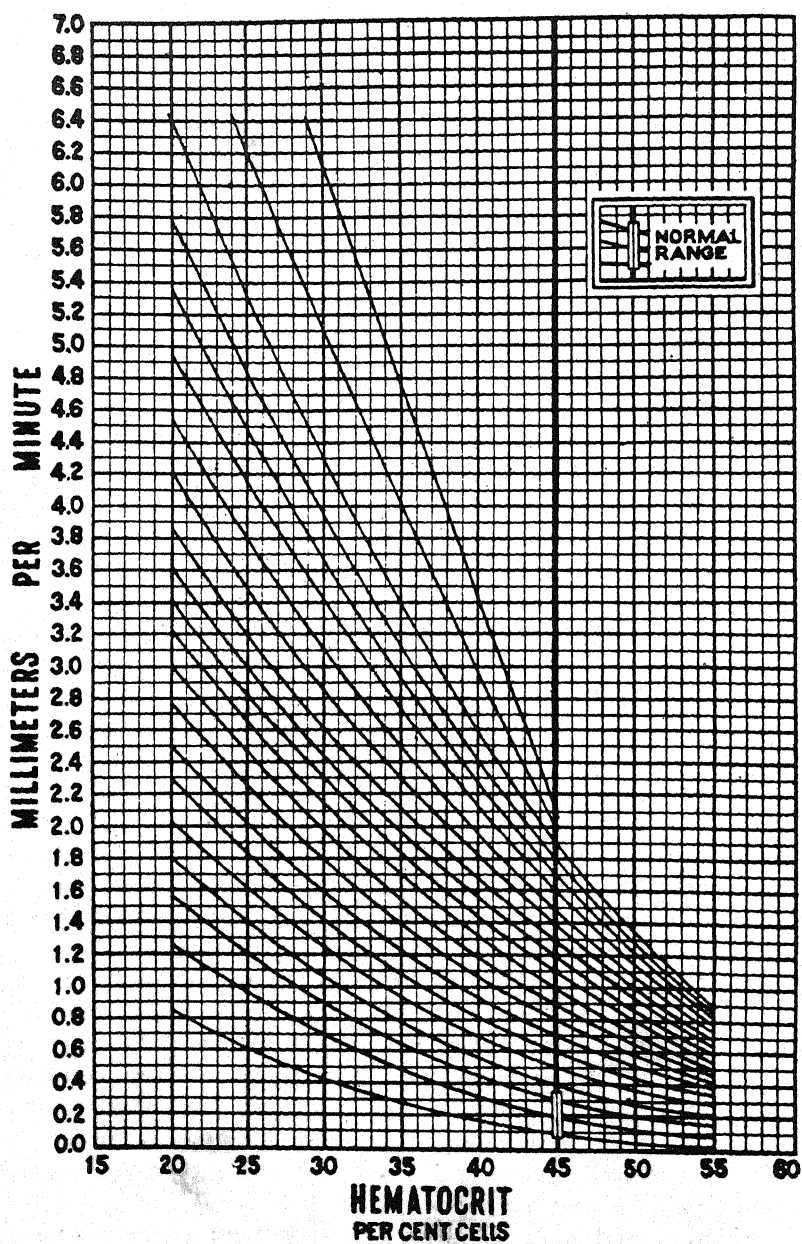


FIG. 5. Chart for calculation of sedimentation rate of red blood cells for varying hematocrit values, in terms of a hematocrit value of 45. (After Rourke and Ernstene.<sup>30</sup>)

by this factor is compensated for if sufficient cells are measured. For extremely careful work it would be necessary to measure both diameters of cells which are not round. A convenient method of recording the sizes is to check off, on a piece of paper opposite a column of figures indicating the sizes, the number of each size of red cells. It is well to measure at least 200 red cells, preferably 500, for each determination. Several areas on the field should be selected. On a film made on a glass slide, there is a tendency for the larger red cells to be gathered at the end of the film. The size of the red cells must be calculated from the number of times the ocular magnifies objects on the stage. For example, if a given red cell is covered by five lines in the ocular micrometer and it has been found that five lines will cover  $6\mu$  on the stage micrometer, then the size of the red cell measured will be  $6\mu$ . If the tube length is changed between observations it is necessary to re-determine the magnification. The data may be plotted on cross section paper with the abscissas representing percentage and the ordinates representing the diameter of the red blood cells in micra.

There are three general types of ocular micrometers: one with a fixed scale in which 5 or 10 mm. is ruled in 0.1 mm. divisions or 0.05 mm. divisions. The second type is the movable scale micrometer which is ruled as in the first type but the scale can be moved by a screw attached to the side of the ocular. This has some advantage over the fixed scale inasmuch as a greater delicacy of movement can be obtained by adjusting the scale than by moving the mechanical stage of the microscope. The third type is the filar micrometer which combines a fixed scale and a movable line. The latter is a wire which can be moved across the field by means of a screw attached to an adjustable drum, the head of which is divided into fifty parts. This type of micrometer is used for very delicate measurements. For measurements of red blood cells it is too refined for clinical use. The ideal normal distribution of sizes is as follows:  $6.0\mu$ , 6.25 per cent;  $6.75\mu$ , 25.0 per cent;  $7.5\mu$ , 37.5 per cent;  $8.25\mu$ , 25.0 per cent;  $9.0\mu$ , 6.25 per cent.

**2. Projection Method of Measuring Red Blood Cells.** The red blood cells may be drawn on a piece of paper using the camera lucida and the magnification obtained by drawing under exactly similar conditions the lines of a micrometer slide. Another method is to project the image of the cells on a screen, using a projection microscope, and measure the cells in a similar way.

**3. Photographic Method of Ponder and Millar.<sup>34</sup>** This method is adapted for either fixed films or fresh blood. The blood is photo-

<sup>34</sup> Ponder, E., and Millar, C. K. *Quart. J. Exper. Physiol.*, 9: 67, 1924.

Dryerre, N. B., Millar, C. K., and Ponder, E. *Quart. J. Exper. Physiol.*, 16: 69, 1926.

graphed, using a condenser working at N.A. = 1 with an objective at N.A. = 1.3. A blue light from a "Point-o-light" is used. Magnification is determined by photographing a calibrated stage micrometer at the beginning and at the end of the observations. The fixed blood film or the slide containing living blood cells under a coverslip sealed with petrolatum is photographed and the cells are measured on the developed plate. Care should be taken to secure cells which are not crenated or injured. Preparations in which there is rouleaux-formation are not suitable. A minimum of 100 cells should be measured, preferably 500 cells, although the probable error of the mean in the former case is only about twice as great as in the latter case, and with careful measurements this error is far less than the experimental error. The average number of cells per plate is about 10, so that numerous plates must be taken from different parts of the field. The cells are measured with a calibrated scale, divided into 0.1 mm., each cell being examined under a low power microscope.

4. **Diffraction Method (Pijper).**<sup>35</sup> The diffraction micrometers of Emmons (erimeter) or Eve (halometer) or a similar mechanism may be used. A smooth, non-stained blood film on a cover glass or slide is required. The film is placed in the instrument and the source of light is viewed through the blood. The edges of the cells diffract the rays, producing a set of circular concentric spectra. The portion of the yellow band is observed (or calculated from the position of the red band) in relation to a fixed point. The average size of the red blood cells can then be read from calibrations on the instrument, or calculated from the data given in published curves or tables.

## XIX. Methods of Studying Hemagglutination and Hemolysis of Blood Cells

1. **Classification and Nomenclature of Blood Groups in Man.** Several methods of naming the blood groups appear in the literature.

*The method of Jansky*<sup>36</sup> has been accepted because of priority. This is as follows:

- Group I. Serum agglutinates cells II, III and IV; cells are not agglutinable.
- Group II. Serum agglutinates cells III and IV; cells are agglutinated by serums I and III.

<sup>35</sup> Pijper, A. *J. Path. & Bact.*, 34: 771, 1931.

Ponder, E. *Brit. J. Exp. Biol.*, 6: 427, 1929.

Eve, F. C. *Lancet*, 1: 1070, 1928.

Emmons, W. F., *Quart. J. Med.*, 21: 83, 1927.

<sup>36</sup> Jansky, J. *Haematol. studie u. psychotku, Sbornik Klinicky*, 8: 85, 1907.

Group III. Serum agglutinates cells II and IV; cells are agglutinated by serums I and II.

Group IV. Serum agglutinates no cells; cells are agglutinated by serums I, II and III.

Or graphically:

Corpuscles	Serum			
	I	II	III	IV
I.....	-	-	-	-
II.....	+	-	+	-
III.....	+	+	-	-
IV.....	+	+	+	-

+ = agglutination  
- = no agglutination.

The method of Moss<sup>37</sup> is as follows:

Group I. Serum is non-agglutinative; cells are agglutinated by serums II, III and IV.

Group II. Serum agglutinates cells I and III; cells are agglutinated by serums III and IV.

Group III. Serum agglutinates cells I and II; cells are agglutinated by serums II and IV.

Group IV. Serum agglutinates cells I, II and III; cells are non-agglutinable.

Corpuscles	Serum			
	I	II	III	IV
I.....	-	+	+	+
II.....	-	-	+	+
III.....	-	+	-	+
IV.....	-	-	-	-

Group I of Jansky corresponds to Group IV of Moss and Group IV of Jansky corresponds to Group I of Moss.

To avoid the error which may come from the numbers when the classification is not specified, it has been proposed<sup>38</sup> that the different groups be designated by the letters O, A, B and AB in place of Groups I, II, III and IV of the Jansky classification and Groups IV, II, III and I of the Moss system.

2. Method of Determining the Group to Which an Individual's Blood Corpuscles Belong. (Clinical). Two stock serums which are sterile and have been kept in a cool place are needed. These are Groups II and III. Blood is drawn from the person to be tested, either from a vein or from a needle puncture of the skin of the finger or ear. Coagulation is prevented by means of sodium citrate (1 c.c. of a 20 per cent aqueous solution for 10 c.c. of blood, or 0.1 c.c. of a 20 per cent aqueous solution of heparin). Two separate drops of blood are placed on a slide

<sup>37</sup> Moss, W. L. *Bull. Johns Hopkins Hosp.*, 21: 63, 1910.

<sup>38</sup> Moss, W. L. *J. A. M. A.*, 88: 1921, 1927.

and a drop of Group II serum is added to one drop of blood and a drop of Group III serum is added to the other drop. These are stirred separately and a piece of broken cover glass is placed on the side of each drop. Over this a grease-free cover glass is placed. This may be sealed with petrolatum or covered with a bell jar to prevent evaporation. The preparation should be examined during the next half hour to note if the cells are uniformly distributed or if they have become clumped into irregular masses. They sometimes become clumped into rouleaux-formations but this does not constitute agglutination for the purposes of this test. If there is no agglutination in either preparation during the course of an hour, the blood is Group I (Jansky). If both preparations show agglutination the blood is Group IV (Jansky). If only the one with Group II serum shows agglutination then the blood is Group III, whereas if only the one with Group III serum shows agglutination the blood is Group II. Agglutination is best observed under the low power of the microscope. When it is very marked it can easily be seen macroscopically. When much blood and serum are available the test may be carried out in a small test tube, using 0.5 c.c. of the serum and 0.5 c.c. of whole blood or a 5 per cent corpuscle suspension in a physiological saline solution.

3. Method of Determining Blood Group When Only Blood of Group II (Jansky) or of Group III (Jansky) is Available.<sup>39</sup> Blood is drawn from the individual to be tested and from an individual of Group II or Group III; some is allowed to clot so that the serum may be obtained and part is collected with sodium oxalate or citrate (final concentration 0.2 per cent of the anticoagulant) and the corpuscles separated from the plasma. On hollow ground slips or under cover glasses supported by pieces of broken cover glass, serum of the individual to be tested is mixed with corpuscles of the known individual, and corpuscles of the individual to be tested are mixed with serum of the known individual. When the known individual is of Group II the following table indicates the group to which the unknown individual belongs (Jansky classification).

Serum	Red Blood Corpuscles	Result			
		+	o	+	o
Group II	Unknown	+	o	+	o
Unknown	Group II	o	o	+	+
Unknown is Group		I	II	III	IV

<sup>39</sup> Brem, W. V. *J. A. M. A.*, 66: 190, 1916.

Guthrie, C. G., and Huck, J. G. *Johns Hopkins Hosp. Bull.*, 34: 37, 1923.

When the known individual belongs to Group III the following table indicates the Jansky group to which the unknown individual belongs.

Serum	Red Blood Corpuscles	Result			
Group III Unknown	Unknown Group III	+	+	o	o
		o	+	o	+
Unknown is Group		I	II	III	IV

Individuals are occasionally found whose blood does not conform to any of the four groups.<sup>40</sup>

4. **Quantitative Measurement of Hemagglutination and Hemolysis.**<sup>41</sup> A drop of blood is drawn to the 0.5 mark in a leucocyte counting pipette and a sterile 0.85 per cent sodium chloride, 0.2 per cent sodium citrate solution is drawn in until the mixture reaches the 11.0 mark. The pipette is thoroughly shaken. A drop of the mixture is expelled and the rest is blown into a small test tube or vial containing a glass bead. This gives a dilution of 20 times. All glassware must be chemically clean. The freshly shaken mixture is then drawn up to the 0.2 mark of an accurately graduated red counting pipette and agglutinating serum is drawn in until the mixture reaches the 1.0 mark. The fluid is not allowed to run up into the mixing chamber of the pipette but is expelled onto a round moat type of hemacytometer slide and is thoroughly stirred with a glass rod, using care not to scratch the ruled surface. This is covered with a grease-free cover glass. To prevent evaporation the slide is placed in a level-bottomed Petri dish which contains a watch glass with water. This may be kept in an ice box or at room temperature. The number of non-agglutinated cells on the hemacytometer slide may be counted at intervals, using the high power dry objective. From these observations the rate of hemagglutination and of hemolysis per hour can be measured. With this method the maximum agglutination in human blood is reached in twenty-four hours at from 18°C. to 20°C. In dogs' blood the maximum is reached in two to four hours. Before the agglutination starts, a red blood cell count may be made from the preparation.

#### 5. Method of Comparing Two Bloods to See if They are Com-

<sup>40</sup> Guthrie, C. G., and Huck, J. G. *Johns Hopkins Hosp. Bull.*, 34: 37; 80; 128, 1923.

<sup>41</sup> Isaacs, R. J. *Immunol.*, 9: 95, 1924.

patible.<sup>42</sup> Sodium oxalate solution, 20 per cent is drawn to the 1.0 mark of white blood counting pipette. Blood is then sucked in to the 1.0 mark, displacing the oxalate solution. The pipette is then filled to the 11.0 mark with normal saline. The mixture is then blown out into a small test tube. Blood is collected in this manner from both of the individuals to be compared. With a pipette (e. g., a white blood counting pipette or a fine glass tube) the following mixtures are made in a hollow ground slide, or on an ordinary glass slip which has been ringed with petrolatum.

- 3 drops from the first individual and 3 drops from the second individual.
- 2 drops from the first individual and 4 drops from the second individual.
- 4 drops from the first individual and 2 drops from the second individual.

These 3 dilutions may be made in duplicate. The contents of the drops are mixed with a fine glass rod and the drops are covered with a cover glass to prevent evaporation. After fifteen to thirty minutes the blood mixtures are examined for agglutination. An even distribution of the cells in all the mixtures means that the bloods are perfectly compatible. If the red cells are clumped in all the mixtures, the individuals are not compatible. If the red cells are clumped only in dilutions No. 1 and No. 2 and very few are clumped in dilution No. 3, then individual No. 2 may be used as a donor in transfusions in case of necessity, as this dilution represents a condition in which the bulk of the solution would correspond to the recipient's serum, and the lesser amount to the donor's blood. Such a condition would correspond to the giving of Group I (Jansky) blood to a patient who belongs to either Group II, III or IV.

## XX. Method of Studying Sickling of Red Blood Cells<sup>43</sup>

Fresh preparations of blood are made by bringing a clean cover slip in contact with a drop of blood from the finger tip or ear and mounting the preparations immediately on a glass slip. The edges are sealed with petroleum jelly. After standing for six hours, about  $\frac{3}{4}$  of the cells will have developed the "sickle" shape or will have assumed other bizarre forms with long thread-like processes extending from the cells. After three days to six weeks the cells will re-assume the spherical form. In preparations from patients with mild symptoms 75 per cent of the cells assume the odd forms and in those from patients without symptoms 25 per cent take on the abnormal shapes. For this test, washed corpuscles

<sup>42</sup> Isaacs, R. *Manual of Clinical and Laboratory Technic*, Ed. 3, Cincinnati, 1927, p. 53.

<sup>43</sup> Huck, J. G. *Johns Hopkins Hosp. Bull.*, 34: 335, 1923.

suspended in physiological salt solution may be used or whole blood which has been oxalated. The results are the same in both cases. Several slide preparations should be made, as occasionally the sickling will fail to appear in one preparation. It may be well to try some preparations having one edge of the cover glass raised by a broken cover slip. This will give layers of blood cells of different thicknesses. If the cover glass is removed after the sickling has appeared, most of the cells assume the round form.

## XXI. Methods of Demonstrating Special Features of Structures in Red Blood Cells

1. **Staining Red Blood Cells for Reticulum.** (Permanent Preparations.)<sup>44</sup> The cover glasses are prepared as given in the technique for Wright's stain. A solution of brilliant cresyl blue (0.3 per cent) in 95 per cent alcohol (filtered) is allowed to dry on some of the cover glasses. A convenient way of applying the cresyl blue is to dip an ordinary microscope slip into the cresyl blue solution and stroke it across the cover glass and the cresyl blue will dry as an even film. The cover glass can then be polished in the usual way, using less pressure than when there is no stain on it. Blood is drawn from the finger tip as previously outlined and a drop about 2 mm. in diameter is taken on one end of a fresh cover glass. The edge of this cover glass is brought into contact with a cover glass on which cresyl blue has been dried, and the glasses are separated, and brought together in hinge fashion to mix the cresyl blue with the blood. The cover glasses are then allowed to touch and after the blood has spread they are rapidly drawn apart. After the films have dried they are counterstained with Wright's stain, using the usual technique. In the final washing the process is not as prolonged as with the Wright stain only, as some of the cresyl blue may be dissolved out of the cells.

With this stain the red cells appear as with the usual Wright's stain technique but the reticulated cells show a blue precipitate of different patterns, depending upon the concentration of the so-called reticulum substances in the cells.

**Reticulocyte Staining; Method of Osgood and Wilhelm.**<sup>45</sup> In a small test tube, 5 drops of oxalated venous or capillary blood are mixed with 1 per cent brilliant cresyl blue in 0.85 per cent sodium chloride solution. After one minute, thin films are made and dried in air. These films may be used within twenty-four hours for counting the

<sup>44</sup> Cunningham, J. H. *Arch. Int. Med.*, 26: 405, 1920.

<sup>45</sup> Osgood, E. E., and Wilhelm, M. M. *J. Lab. & Clin. Med.*, 19: 1129, 1933-34.



relative percentage of reticulocytes, or they may be counterstained with Wright's stain, and permanent mounts made. All red cells and reticulocytes in adjacent fields (500 to 1000) are counted and the percentage of reticulocytes is noted.

2. **Criteria for Determining the Relative Amount of Hemoglobin in the Cells Using a Stained Blood Film.** In a film stained with Wright's stain, the amount of red in the nuclei of the white blood cells is noted. If the red blood cells are faintly stained while the white cells have taken up a considerable amount of the eosin element of the stain, the red blood cells are *hypochromic*. If they have taken up a very great amount comparatively, they are *hyperchromic*. The normal condition, *orthochromic*, is best determined by the study of the staining qualities of the red blood cells of a healthy individual. The presence of a light area in the center of the cell does not necessarily mean hypochromia. Many parts of the blood film should always be studied before conclusions are drawn.

3. **Malarial Parasites.** The parasites are best studied in a drop of fresh blood mounted under a petrolatum ringed cover glass. They are recognized in the red blood cells by their granules, which are highly refractive and in active movement. The parasites stain well with the ordinary Romanowsky type of stains. For some cases fairly thick blood films are advantageous.

4. **Diffuse Basophilia, Polychromatophilia.** Romanowsky type of stains (eosin-methylene blue combinations, Wright's, Leishman's, Jenner's stains). The basophilic red blood cells stain grayish or slightly more blue than the normal red blood cells. Occasionally the distribution of the color is irregular in the corpuscle and both reddish and bluish areas are present (polychromatophilia). Both manifestations are evidence of youth in the corpuscles. The bluish staining substance is probably the same substance which precipitates as a reticulum with brilliant cresyl blue used supravitaly.

5. **Stippling.** The stippling of red blood cells shows as fine bluish black dots in the corpuscles with Wright's and similar stains. The spots can be demonstrated with methylene blue alone, and are best seen in thick blood films. The method of Aub and his colleagues<sup>46</sup> is as follows:

Fix thin cover glass films of blood with methyl alcohol, and allow to dry. The stain used is methylene blue, 1 gm.; potassium carbonate, 1 gm.; in 100 c.c. of distilled water. This is the stock solution. A dilution of one part of the stain and 14 parts of water is placed over the blood for fifteen minutes. The excess stain is washed off with water until the blood film appears bluish green. The film is dried and the blood is examined using the oil immersion lens. The

<sup>46</sup> Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P. *Medicine*, 4: 1, 1925.

stippled cells are easily located. They are somewhat more polychromatic than the normal, light greenish blue erythrocytes. The basophilic granules appear dark blue, as do the nuclei of the leucocytes.

**Methods of McCord, Holden and Johnston.**<sup>47</sup> Thin even films of blood are made on glass slides and are allowed to dry between one and three hours. Then one-half of the film is covered with a piece of filter paper and this is carefully moistened with methyl alcohol. When it dries, the paper becomes loose and can be removed. In this way only one-half of the film is fixed; so that the cells in the unfixed portion will hemolyze during the staining process. The slide is stained in a Coplin jar for ten minutes, using a modified Manson's methylene blue. (Borax, 1 gm., methylene blue 2 gm., boiling distilled water, 100 c.c. The borax is dissolved in the water and the methylene blue is added to this. It is filtered, and remains stable for at least two weeks.) After staining, rinse 3-4 times in distilled water and dry in air.

Stippled cells (basophilic aggregations) are counted in 20 fields of the unfixed section of the slide and the number of red blood cells in a corresponding number of fields (5 fields  $\times$  4) is determined from the fixed area. The percentage of stippled cells is then determined by dividing by the number of stippled cells (or basophilic aggregates)  $\times$  100 by the total number red cells counted per similar number of fields.

**6. Nuclei, Nuclear Particles, Howell-Jolly Bodies.** These structures are well shown with all the commonly used blood stains. They all take on the characteristic color of the nuclei of the other nucleated cells.

**7. Refractive Granule.** This structure<sup>48</sup> can be easily seen in about 1 per cent of the red blood corpuscles of normal human beings and mammals (increased in number under some conditions) in fresh blood, or fresh blood with a supravital stain, in unstained dried films, or in films stained with any of the commonly used stains. The granule is about 0.5 micron in diameter, highly refractive and does not stain. It appears as colorless or black, depending on the focus, or it may be tinged with the same or complementary color of the surrounding solution or stain. The granule is an evidence of youth and probably represents a stage in maturation of the red blood corpuscle between that of the reticulocyte and the adult corpuscle.

**8. Cabot's Rings.** These stain as red filaments (circular or figure eight) with most of the Romanowsky stains. The color of the red blood corpuscle included inside of the ring is the same as that outside of this

<sup>47</sup> McCord, C. P., Holden, F. R., and Johnston, J. *Am. J. Public Health*, 25: 1089, 1935.

<sup>48</sup> Isaacs, R. *Anat. Rec.*, 29: 299, 1925.

structure, differentiating it from some artifacts which may cause confusion in identifying it.

## XXII. Blood Platelet Diluting Solutions

### 1. Buckman and Hallisey.<sup>49</sup>

Glucose.....	6.0 gm.
Sodium citrate.....	0.4 gm.
Distilled water.....	100 c.c.

Filter and add 0.02 gm. of toluene red (dimethyldiamidotoluphenazin). To this is added 0.1 gm. of crystal violet. The mixture is gently heated to 60°C. and held at this temperature for five minutes and allowed to cool to room temperature. It is then centrifugalized for ten minutes at 2000 revolutions per minute. The supernatant liquid must be filtered twice through three thicknesses of dry filter paper (No. 30 Whatman). This solution is preserved by 0.2 c.c. of formaldehyde solution. It keeps indefinitely.

### 2. Kristenson.<sup>50</sup>

Urea.....	10 gm.
Sodium citrate.....	2.5 gm.
Corrosive sublimate.....	0.005 gm.
Brilliant cresyl blue.....	0.5 gm.
Distilled water.....	500 c.c.

### 3. Ottenberg and Rosenthal.<sup>51</sup>

Sodium citrate.....	3 gm.
Distilled water to 100 c.c.	

Cresyl blue 1 to 500 or methyl violet 1 to 500 may be added freshly to the solution just before using, and the solution then filtered.

### 4. Pratt.<sup>52</sup>

Sodium metaphosphate.....	2.0 gm.
Sodium chloride.....	0.9 gm.
Distilled water.....	100 c.c.

### 5. Rees and Ecker.<sup>53</sup>

Sodium citrate.....	3.8 gm.
Distilled water.....	100 c.c.
Formaldehyde solution.....	0.2 c.c.
Brilliant cresyl blue.....	0.1 c.c.

<sup>49</sup> Buckman, T. E., and Hallisey, J. E. *J. A. M. A.*, 76: 427, 1921.

<sup>50</sup> Kristenson, A. *Acta Med. Scandinav.*, 57: 301, 1922.

<sup>51</sup> Ottenberg, R., and Rosenthal, N. *J. A. M. A.*, 69: 999, 1917.

<sup>52</sup> Pratt, J. H. *J. A. M. A.*, 45: 1999, 1905.

<sup>53</sup> Rees, H. M., and Ecker, E. E. *J. A. M. A.*, 80: 621, 1923.

6. Wright and Kinnicutt.<sup>54</sup>

Solution I. Brilliant cresyl blue..... 1.0 gm.

Distilled water..... 300.0 c.c.

The growth of yeast may be prevented by keeping the solution in an ice box.

Solution II. Potassium cyanide..... 1.0 gm.

Distilled water..... 1400.0 c.c.

The solutions are kept in separate bottles and just before using, two parts of the brilliant cresyl blue solution are mixed with three parts of the potassium cyanide solution and the fluid filtered.

## 7. Toisson.

Distilled water..... 160 c.c.

Glycerin, neutral..... 30 c.c.

Sodium sulphate..... 8 gm.

Sodium chloride..... 1 gm.

Methyl violet..... 0.025 gm.

8. Hayem.<sup>55</sup>

Distilled water..... 200 c.c.

Sodium chloride..... 1 gm.

Sodium sulphate..... 5 gm.

Mercuric chloride..... 0.5 gm.

## 9. Gower.

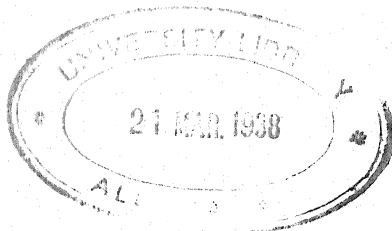
Sodium sulphate..... 7 gm.

Acetic acid..... 4 c.c.

Distilled water to 120 c.c.

<sup>54</sup> Wright, J. H., and Kinnicutt, R. *J. A. M. A.*, 56: 1457, 1911.

<sup>55</sup> Hayem, G. *Arch. de Physiologie*, 5: 692, 1878.



# METHODS FOR THE STUDY OF LEUCOCYTES

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METHODS FOR CELLS OF THE CIRCULATING BLOOD 324. OBTAINING BLOOD 324. DRY FILMS 325. Preparation 325. Fixation and staining methods 325. WET FILMS. Fixation and Stains 331. METHODS FOR PERITONEAL FLUID 332. METHODS FOR THE PEROXIDASE TEST 332. Goodpasture's stain 332. Graham's benzidine stain 333. METHODS FOR MEGACARYOCYTES AND BLOOD PLATELETS 333. WRIGHT'S MEGACARYOCYTE STAIN 334. KINGSLEY'S STAIN 335. Technique 335. METHODS FOR FIXED TISSUE LEUCOCYTES 336. CELLS OF THE CONNECTIVE TISSUE 336. Preparation of subcutaneous tissue spreads 336. Preparation of omentum and mesentery spreads 336. Preparation of body-wall for sectioning 337. Fixation and stains for connective tissue cells 337. CELLS OF THE BONE MARROW, LYMPH NODES AND SPLEEN 337. Fixation of wet smears 337. Fixation for sectioning 338. Bone marrow biopsy 338. Stains 339.

## A. METHODS FOR CELLS OF THE CIRCULATING BLOOD<sup>1</sup>

### I. Obtaining Blood

Human blood is taken from a finger or a lobe of the ear. If the finger is used, Naegeli<sup>2</sup> recommends that the entire hand be immersed in warm water until suffused and then dried with chafing, in order to secure a free flow of blood and thus an even distribution of cells. In working with infants the great toe or the heel is used. Rabbits bleed freely if a puncture is made in the ear, preferably through the marginal vein. The ear should be shaved. In animals which are excited the vein may be contracted so that little blood flows, but a dilation of the vein may be caused instantly by wiping the spot with xylene. Guinea pigs, also, may be bled from the ear; but in this case a simple puncture may not be sufficient, and it is usually better to cut a slit in the ear. When it is necessary to secure much blood or to study that which is not peripheral, a method of ventricular puncture may be used, puncturing the heart with a paraffined or oiled needle. This is easily done on the larger animals: dogs, rabbits, and even guinea pigs. The easiest method with rats is to cut off a piece of the tail. With salamanders, also, the tail is used, and it should be well scrubbed just before taking the blood in order to get rid of the mucus. Blood taken in this way is mixed with a small amount of tissue fluid. It should be kept from contact with torn

<sup>1</sup> Methods for counting leucocytes and for supravital staining will be found on pp. 111, 292.

<sup>2</sup> Schridde, H., and Naegeli, O. *Die Hämatologische Technik*. Jena, 1921.

tissue or with the skin as much as possible. If the life of the animal can be sacrificed, purer blood can be obtained by opening the chest and taking it from the heart with a needle. In the case of birds a wing vein in the axillary region may be used, but since this causes subcutaneous bleeding the tibial vein in the ankle is preferable. A method is given by Nolf for obtaining blood from fishes without letting it touch the skin. Hold the fish in a vertical position with the head down and cut off the tail back of the dorsal fin. Run a glass cannula coated with paraffin into the dorsal aorta. Then place the fish in a horizontal position, and the blood will run out. A more usual method is to take it from the heart. The method of getting blood directly from the heart with a lip pipette is useful for embryos and small animals. It is the best method for frogs. The lip pipette described by Fry<sup>3</sup> is a glass pipette with one end drawn to a suitable fineness and fitted at the other end with a rubber tube with a glass mouthpiece. If the end of the tube is of capillary fineness so that the heart is not torn, blood will remain unclotted for a few minutes. As compared with a syringe needle it has the advantage of cheapness since the capillary tubes can be used freely and then thrown away. A glass tube is not successful unless it is of capillary fineness. A more extended account of methods for securing blood from various kinds of animals is given by Jolly.<sup>4</sup>

## II. Dry Films

1. **Preparation.** The preparation of blood films by the smear method is described in Part I (pp. 33, 34). Blood films may be made on glass slips, but the cover glass method is better for leucocytes. They should be fairly thin in order to demonstrate granules, especially azure or neutrophile granules. According to most authors a blood film can be more successfully stained about twenty-four hours after it has been made than when perfectly fresh. It should not, as a rule, be older than one week, although the time within which good results may be secured varies according to conditions, possibly atmospheric. Fixation and staining may be accomplished in one series of operations if an alcoholic stain is used, or the fixation may be done first, followed by a staining process.

2. **Fixation and Staining Methods.** Most blood stains contain methylene blue, methylene azure, methylene violet and eosin. They are discussed at length by MacNeal.<sup>5</sup> Since Romanowsky was the first to point out the selective action of such stains, mixtures containing these

<sup>3</sup> Fry, H. J. *Anat. Rec.*, 34: 245, 1927.

<sup>4</sup> Jolly, J. *Traité d'Hématologie*. Vol. 1, Paris, 1923.

<sup>5</sup> MacNeal, W. J. *J. Infect. Dis.*, 3: 412, 1906.

dyes are often spoken of as Romanowsky stains and the red lavender color produced by them in the nuclei of lymphocytes and monocytes is known as the Romanowsky effect. There are a number of modifications. Leishman's, Wilson's and Wright's are similar, all being eosinates of polychromed methylene blue. Giemsa's modification contains the dyes called azure II-eosin and azure II; but since azure II is a mixture in equal parts of methylene azure and methylene blue, the stain is really a mixture of the eosinates of methylene blue and methylene azure. These stains contain methylene violet only as an impurity of the methylene blue. Newer stains have more exact formulas which give definite weights for each dye present. Pappenheim's modification<sup>6</sup> is called "panchrome" and contains the following dyes: methylene blue, toluidin blue, azure I (methylene azure), methylene violet and eosin. Pappenheim gives several percentage formulas for making it. It can be obtained from the Grübler Company, but the proportions which that company uses for it are not known. Of the newer stains MacNeal's tetrachrome is the best known. It contains methylene blue, methylene violet, methylene azure A and eosin. Kingsley's stain (see megacaryocytes) may also be used on blood, giving an effect similar to Wright's. Giemsa, Wright's and the tetrachrome stain may be obtained from American companies with the certification of the Commission on Standardization of Biological Stains.

a. *Wright's Blood Stain.*<sup>7</sup> (1) Technique. Wright's blood stain may be used on any dry smear. The mixture fixes and stains at the same time. For convenience in handling, the stain can be confined to a part of the slide by marking across one end with a piece of soft paraffin. The slip or cover glass should be level. In working with cover glasses it is well to support them by cover glass holders or simply on the ends of small corks. By means of a pipette the preparation is quickly flooded with enough stain to cover it well. A 22 mm. cover requires 8 or 10 drops, a slide 30 or 40 drops. The stain is left on for sixty seconds and as it is dissolved in absolute methyl alcohol, the blood is fixed during this interval. An insufficient amount of stain causes a round granular precipitate by evaporation of the alcohol. Then with a second pipette distilled water is added drop by drop until a greenish metallic scum begins to form. This usually happens when the distilled water equals about half the amount of stain. The slide should be moved gently while the water is being added in order to insure rapid mixing of the water and stain. The diluted stain is left on for two and one-half to three minutes. It is then rinsed off with a pipette of distilled water and, keeping the slide

<sup>6</sup> Pappenheim, A. *Folia haematol.*, Archiv, 11: 194, 1911.

<sup>7</sup> Mallory, F. B., and Wright, J. H. *Pathological Technic*. Phila., 1924.

level, fresh distilled water is added. This is left another two and one-half minutes and rinsed off. Finally the slide is carefully blotted dry with smooth filter paper. By following a scheme such as this fairly uniform results can be obtained with normal blood. However, variations in the type of blood or the thickness of the film may affect the result, making advisable slight changes in timing. A preparation may be dried and examined at leisure, and then, if it is found to be too dark, it can be further washed with distilled water; but if it has been washed until too light, first class results cannot be obtained by restaining it. If restaining is necessary, it is best to decolorize the slide first. Nuclear structures and the azurophil granules of platelets, lymphocytes and monocytes should be distinct. When thoroughly dry the preparation can be mounted with neutral balsam or with damar.

(2) Preparation of Stain and Use of Buffer Solution. If the stain is obtained as a powder, both Grüber and American products can be made up in the proportion of 0.3 gm. to 100 c.c. of pure methyl alcohol. The solution should be shaken occasionally and is ready for use in three or four days. Detailed directions for making the powder are given by Wright. The solution is not indefinitely stable, however, as it may grow more concentrated by evaporation of the alcohol and require dilution, or contamination with moisture may precipitate it or make it liable to precipitation on the slide, or it may undergo slow oxidative changes. Fresh distilled water is necessary for good results. It should be slightly acid and according to Haden<sup>8</sup> it may have a pH of 6.0 to 6.6. This is easily tested with phenol red or brom thymol blue. If it stands for a week or so in an ordinary glass bottle, it usually becomes unfit for use. Buffer solutions may be used instead, however, and are stable. The McJunkin-Haden buffer is satisfactory and has a pH of 6.4. It is made according to the following formula:

Monobasic potassium phosphate.....	6.63 gm.
Anhydrous dibasic sodium phosphate.....	2.56 gm.
Distilled water.....	1 liter

This solution can be used all through the technique of blood staining to replace distilled water in diluting the stain.

*b. MacNeal's Tetrachrome.*<sup>9</sup> This stain is sold as a solution or as a dry mixture of dyes. Dissolve 0.5 gm. of the dry ingredients in 100 c.c. methyl alcohol, neutral, acetone free by adding small portions to the alcohol warmed to 50°C. and shaking between each addition. Leave at 37°C. for two days with occasional shaking and filter. It was pointed out

<sup>8</sup> Haden, R. L. *J. Lab. & Clin. Med.*, 9: 64, 1923.

<sup>9</sup> MacNeal, W. J. *J. A. M. A.*, 78: 1122, 1922.



by Conn<sup>10</sup> that unless these directions are followed, the solution does not keep well.

Use on blood films like Wright's stain.

c. *Giemsa*.<sup>11</sup> (1) After Methyl Alcohol Fixation. For ordinary dry films the simplest fixation is alcohol. Most directions call for a three minute methyl alcohol fixation of fresh films and a two minute fixation of films twenty-four hours old. This should be done in a covered dish, and the slide should be taken out and allowed to dry before it is stained.

The alcohol fixation is followed by Giemsa's stain for fifteen minutes, after which the preparation is washed with distilled water until properly differentiated and then blotted dry. Giemsa's stain should be prepared immediately before use in the proportion of 1 drop of the stock solution to 1 c.c. of distilled water or buffer with a pH of 6.4 to 6.8. Giemsa stock solution according to the proportions given for Grüber dyes:

Azure II-eosin.....	0.6 gm.
Azure II.....	0.16 gm.
Methyl alcohol, pure and free from acetone.....	50.00 c.c.
Glycerin, pure and free from water.....	50.00 c.c.

Dissolve the dyes in the glycerin at 60°C. and then add the alcohol warmed to 60°C. Certified Giemsa stock solution can be obtained from a number of American companies. The product of the Gradwohl Laboratories stains blood in seven to eight minutes when used in a dilution of 3 drops to 2 c.c. of distilled water. After about thirty minutes the stain begins to precipitate. With Giemsa's stain and its modifications it is well to avoid the danger of a precipitate forming on the film by floating a cover glass preparation on the stain or supporting a slide in a dish of stain with the smeared side down. With good stains and pure distilled water precipitation should not occur within the time of staining. Giemsa gives the same differential stain of blood elements as is obtained by the well-known Wright's or Leishman's solution. It is better for azure granules. Neutrophile granules are usually rather pale, especially if the film is at all thick. It is an adaptable stain; variations can be made in the time of staining, in the dilution of the stain, and in the hydrogen ion concentration of the diluted stain.

(2) After May-Grünwald Fixation. May-Grünwald is a saturated solution in methyl alcohol of the eosinate of methylene blue. It contains the useful azure only as methylene blue contains some methylene azure. Used alone it affords a weak, unsatisfactory stain, but it improves the appearance of nuclei and is a more dependable fixation for Giemsa than

<sup>10</sup> Conn, H. J. *Stain Technology*, 2: 31, 1927.

<sup>11</sup> Giemsa, G. *Centbl. f. Bakt., I Abt.*, 37: 308, 1904.

alcohol. Jenner's stain<sup>12</sup> is identical with May-Grünwald and may be used instead.

The May-Giemsa or Jenner-Giemsa combination of Pappenheim<sup>13</sup> is the popular stain for blood films in most European laboratories. It can also be used for sections. Fix by covering the air-dried film with May-Grünwald stain for three minutes. Add an equal amount of distilled water and leave the diluted stain for one minute. Pour off and without rinsing cover with diluted Giemsa solution. Leave for fifteen to twenty minutes or less according to dilution. Rinse off the stain and leave the slide in pure distilled water for about one minute or until differentiated. Blot dry and mount.

(3) After Acetone-lucidol Fixation. The acetone-lucidol method of St. Szécsi<sup>14</sup> also gives a good fixation of dry films. Lucidol is a proprietary name for benzoyl peroxide, a white powder which explodes when heated to melting. Fix for fifteen minutes in a covered dish of benzoyl peroxide solution (10 gm. benzoyl peroxide, 100 c.c. acetone). Transfer without drying to a covered dish of acetone-xylol (acetone 3 c.c., xylol 2 c.c.) for ten minutes to dissolve the benzoyl peroxide crystals. Place in methyl alcohol for one-half to one minute. Stain in May-Giemsa, about five minutes in May-Grünwald diluted with distilled water and about fifteen minutes in Giemsa. The method shows azure granules well.

*d. Panchrome.* Pappenheim's panchrome<sup>15</sup> is a modified Giemsa stain. The powder obtained from the Grüber Company and made up according to their directions is brought into solution by adding 0.75 gm. to 75 gm. of methyl alcohol (pure and free from acetone) and 25 gm. of glycerin (acid-free) at 60°C. After being cooled it is filtered through a dry filter and kept well stoppered. It is used with May-Grünwald according to the directions given for Giemsa. The result is a better demonstration of neutrophile granules and better metachromasia of mast granules than the Giemsa solution gives, but in our experience some delicacy of stain is lost, and the cells are more likely to be muddy.

*e. The Kardos-Pappenheim Modification.* This is also intended to make neutrophile granules more evident. Fix the film with May-Grünwald for three minutes. Dilute with an equal volume of distilled water and leave for one minute. Pour off the solution and stain with Giemsa methyl green-orange mixture for fifteen minutes. Wash briefly in water, dry, and mount.

<sup>12</sup> Jenner, L. *Lancet*, Pt. 1, p. 370, 1899.

<sup>13</sup> Pappenheim, A. *Folia haematol.*, Archiv, 22: 15, 1917.

<sup>14</sup> Szécsi, S. *Deutsche med. Wchnschr.*, 39: 1584, 1913.

<sup>15</sup> Pappenheim, A. *Folia haematol.*, Archiv, 11: 194, 1911.

The Giemsa methyl green-orange stain is made, according to Kardos,<sup>16</sup> as follows: 2 per cent aqueous orange G solution is mixed with concentrated aqueous methyl green. The precipitate is dried and dissolved in methyl alcohol. The stain is made by taking 10 drops of Giemsa, 5 drops of methyl green-orange, and 15 c.c. of distilled water. It should be thoroughly shaken and poured from the mallow colored foam. Panchrome can also be modified in this way. The attempt is to combine the effect of triacid, the best stain for neutrophile granules, with that of May-Giemsa, the best stain for azure granules. Kardos states that with his modification a number of cells would be called neutrophilic promyelocytes, which with Giemsa alone would be called non-granular leucoblasts.

f. *Triacid*.<sup>17</sup> Ehrlich's triacid is useful as a check on the Romanowsky stains because it colors neutrophile granules but not azure granules. It has been repeatedly stated by Ehrlich and Pappenheim that no conclusions should be drawn as to the presence or absence of neutrophile granules unless this test has been applied. Ehrlich used heat fixation of 110°C. for one-half to two minutes. A copper plate is heated by a bunsen burner at one end, and the air-dried film is placed between the points where toluene (B.P. 110°C.) boils and water assumes the spheroidal state, 150°C. Other fixations are methyl alcohol twenty minutes or acetone five minutes. The triacid stain is used for five minutes. Then wash in distilled water until no more color comes away, dry and mount.

Triacid contains methyl green, orange G and acid fuchsin. A satisfactory stain is difficult to prepare, probably because methyl green is not a stable compound, either in a powdered form or in solution. It is a saving of time, therefore, to obtain it, as well as the above mentioned methyl green-orange solution, ready made from the Grüber Company.

g. *Stains for Toxic Neutrophiles*. Pathologic changes in neutrophiles can be seen with the usual May-Giemsa stain, toxic granules appearing larger and darker in color than normal granules. Mommsen pointed out in 1927 that normal neutrophile granules stain at a pH of 6 to 7, while at a pH of 5.4 toxic granules stain and normal granules remain unstained.

(1) Method of Mommsen.<sup>18</sup> Fix the air-dried blood film in absolute methyl alcohol for four minutes. It is recommended that the slide be left for twenty-four hours in an incubator at 37°C. to equalize drying. Stain in buffered Giemsa solution for one hour. The stain is made

<sup>16</sup> Kardos, E., *Folia haematol.*, Archiv, 12: 39, 1911.

<sup>17</sup> Ehrlich, P., and Lazarus, A. *Die Anaemie*, I Abt. Ed. 2, Vienna, 1909, p. 31.

<sup>18</sup> Mommsen, H. *Ztschr. exper. Med.*, 65: 299, 1929.

by diluting 10 c.c. of filtered Giemsa stock solution (Hollborn, Leipzig) with 40 c.c. of distilled water and adding a buffer solution adjusted to pH 5.4 to make up 100 c.c. Wash the preparation with buffer and blot dry. Old films are harder to stain and may take two hours.

A buffer of 5.4:

Sodium hydroxide N/1.....	26.6 c.c.
Acetic acid N/1.....	27.0 c.c.
Distilled water to.....	1000.0 c.c.

(2) Method of Freifeld.<sup>19</sup> Prepare 2 solutions.

Solution A. Ziehl's carbol-fuchsin.

Solution B. Methylene blue (1 per cent aqueous).

To 20 c.c. of tap water add 7 drops of solution A and 5 drops of solution B. Fix the preparation with methyl alcohol and stain for one hour. Neutrophile nuclei stain blue, basichromatin dark blue and oxychromatin reddish blue. Cytoplasm stains light red. Basophilic granules are dark blue. Toxic neutrophiles have a basophilic cytoplasm in which dark basophilic granules stand out clearly.

### III. Wet Films

**I. Fixation and Stains.** For amphibian blood in which the cells are large enough to be distorted by drying, a fixation of the moist film by vapor is to be recommended. Osmic acid 1 per cent should be used for twenty or at most thirty seconds by the following method. Support the clean slip or cover glass over a dish of 1 per cent  $\text{OsO}_4$  for a few seconds. Make the film in the usual way on the surface that was exposed to the fumes, and put the moist preparation back over the fixative for twenty seconds. Then let it dry. Overfixation will make it stain too intensely. The appearance of nuclear structures is sometimes improved by the addition of glacial acetic acid to the osmic solution, 2 drops to 1 c.c. Excess acetic makes the blood colorless. Formalin vapor may be used instead of osmic for forty seconds to one minute. Formalin and osmic acid are the most common reagents for this purpose, but Weidenreich<sup>20</sup> mentions others. In any case the preparation should be allowed to stand twenty-four hours or more before it is stained. Giemsa's stain is good after this fixation. If Wright's stain is used, the sixty second interval of concentrated stain should be omitted. Any stain containing methylene blue colors red blood cells a greenish blue with this tech-

<sup>19</sup> Naegeli, O. *Blutkrankheiten und Blutdiagnostik*. Berlin, 1931, p. 19.

<sup>20</sup> Weidenreich, F. *Die Leucocyten und verwandte Zellformen*. Wiesbaden, 1911.

nique. If it is desirable to stain them pink, Delafield's hematoxylin and eosin may be used. In this case bluish eosin is preferable to yellowish.

#### B. METHODS FOR PERITONEAL FLUID

This fluid may be preserved by dry films made by the cover glass method (p. 33). The usual blood stains are used on such preparations.

An agar-osmium method was first used by Deetjen for blood platelets, but according to Weidenreich it is useful for all leucocytes. The writers have found it a valuable method for peritoneal fluid. It has the advantage that cells are fixed without being rolled between cover glasses or dragged over a slip. The following account is taken from Weidenreich:

Make a 1 per cent solution of agar in 0.8 per cent salt solution. Fill several test tubes with this to a height of about 1 inch, plug them with cotton, and sterilize them. To use, heat one of the tubes in hot water until the agar has become fluid and pour it out on a glass plate. After the agar has solidified, cut squares from it slightly smaller than the cover glass to be used. Place one of the agar squares on a slide. Then place a drop of peritoneal fluid or blood on a cover glass and lay the cover glass carefully on the agar square so that its edges project beyond the edges of the agar. The fluid spreads out in a thin layer between the glass and the agar. Leave the preparation for five or ten minutes and then add a few drops of 1 per cent osmic acid under the projecting edges of the cover glass but without touching the cover glass itself. After five minutes, carefully raise the cover glass and place it in distilled water for a few minutes. Stain in Giemsa without letting it dry. After staining, rinse the preparation in distilled water, dehydrate in 95 per cent alcohol and 100 per cent alcohol, clear in xylene and mount. Do not let it dry at any stage. This method is of value for mammalian material.

#### C. METHODS FOR THE PEROXIDASE TEST

This reaction depends on the presence of an oxidizing ferment in cells of the myeloid series, definitely distinguishing them from cells of the lymphocyte series and from tissue cells.

1. **Goodpasture's Stain.**<sup>21</sup> Cover a dry blood film with a measured amount of stain and leave for one minute. Add an equal amount of water and leave three or four minutes. Rinse well in water, blot dry and examine with oil. Nuclei are clear red; cytoplasm and platelets stain pale pink; erythrocytes are buff color. Sharply defined blue oxidase granules are found in neutrophils, eosinophils, myelocytes and, in smaller number, in monocytes. They are not found in lymphocytes.

<sup>21</sup> Goodpasture, E. W. *J. Lab. & Clin. Med.*, 4: 442, 1919.

The reaction of myeloblasts is not positive. It is always questionable whether immature cells which show oxidase granules are not early myelocytes instead of myeloblasts.

Stain:

Alcohol.....	100.0
Sodium nitroprusside.....	0.05
Benzidine C. P. ....	0.05
Basic fuchsin.....	0.05
Hydrogen peroxide .....	0.5

Dissolve the nitroprusside in 1 or 2 c.c. of water, mix with alcohol and add the other ingredients. Made in this way the stain is good only a few days. Beacom suggested that if it is made up without hydrogen peroxide, it will keep six to eight months. For use, add 2 drops hydrogen peroxide to 15 c.c. distilled water and dilute the stain on the slide with this solution.

Both this stain and the following one may be used on frozen sections of formalin material.

**2. Graham's Benzidine Stain.**<sup>22</sup> Fix the fresh film a few seconds with a fresh mixture of one part of 40 per cent formaldehyde and 9 parts of 95 per cent alcohol. Lightly wash with water and stain with benzidine solution. Benzidine solution: add a few crystals of benzidine C. P. to 0.02 c.c. of hydrogen peroxide (use Sahli pipette). To this add 10 c.c. of 40 per cent alcohol. After five minutes wash the slide thoroughly under the tap and counterstain for one-half to one minute in aniline water thionin. Thionin stain: add 10 c.c. of saturated thionin in 75 per cent alcohol to 40 c.c. of aniline water.

When staining is completed, oxidase granules are various shades of brown. In neutrophiles they are fine and numerous. In eosinophiles they are coarser. Monocytes usually have a few. Lymphocytes and lymphoblasts have none. Myelocytes show numerous granules. In the most immature cells, however, results are not certain, so that this test does not distinguish surely between myeloblasts and lymphoblasts.

#### D. METHODS FOR MEGACARYOCYTES AND BLOOD PLATELETS

Methods for counting blood platelets are described by Isaacs (p. 293).

For a histological examination the Romanowsky stains are good. Smears treated with Wright's blood stain, Giemsa, or the modifications of Giemsa show a sharp differentiation of the characteristic azurophil granules in platelets and megacaryocytes, no matter whether they are

<sup>22</sup> Graham, G. S. *J. Med. Research*, 39: 15, 1918.

used on the ordinary dry smears or on those fixed as quickly as possible while still moist.

In sectioned material, Schridde was the first to demonstrate the granules of megacaryocyte cytoplasm using azure II-eosin followed by dehydration in acetone. Shortly after this, Wright worked out a modified Romanowsky combination.

### 1. Wright's Megacaryocyte Stain.<sup>23</sup>

Fix in a saturated solution of mercuric chloride in 0.9 per cent salt solution. Dehydrate in alcohol followed by acetone, clear in thick cedar oil followed by xylene, and imbed in paraffin.

Stain sections for ten minutes in a mixture of equal parts of staining fluid and distilled water, poured directly on the slide. A yellowish metallic scum should slowly form, but the stain should not precipitate. The time may be varied. When the cytoplasm of the giant cells is bright red and reticular fibrils begin to look red, the staining should be stopped, and the preparation should be at once washed in water. Dehydrate in pure acetone and clear in pure oil of turpentine. Mount in a thick solution of colophonium in pure oil of turpentine. Before mounting carefully remove superfluous turpentine as it may absorb moisture and cause clouding or fading. Downey<sup>24</sup> found that turpentine-damar works equally well.

Make the stain by taking 3 parts of polychrome methylene blue solution and 10 parts of 0.2 per cent solution of eosin water soluble yellowish (W.G.) in methyl alcohol. For the polychrome methylene blue solution dissolve 1 gm. of methylene blue B.X. in 100 c.c. of a 0.5 per cent aqueous solution of  $\text{NaHCO}_3$  and keep the solution at 100°C. in a steam sterilizer for one and one-half hours. Filter the solution when it is cool. The filtrate is polychrome methylene blue.

Preparations stained in this way show both platelets and megacaryocytes in good detail. Granules are clearer if they are examined by light from an electric bulb which has a yellowish tint. In order to secure satisfactory results, it is absolutely necessary to follow Wright's technique in detail, including the use of acetone and turpentine.

Downey found that the corrosive sublimate fixation of Wright's method, although excellent for megacaryocytes and platelets, was poor for lymphocytes in the same preparation. He substituted a mixture of 10 c.c. commercial formalin and 90 c.c. of 0.9 per cent salt solution, saturated with  $\text{HgCl}_2$ . The solution gives a good fixation of lymphocytes as well as megacaryocytes, and allows not only a typical Wright's stain but also a fairly good stain with the Giemsa and Dominici mixtures.

<sup>23</sup> Wright, J. H. *J. Morphol.*, 21: 263, 1910.

<sup>24</sup> Downey, H. *Folia haematol., Archiv*, 15: 25, 1913.

2. **Kingsley's Stain.**<sup>25</sup> This is a recent stain which produces megacaryocyte and platelet preparations surpassing those of older methods. Fixation is preferably by means of the formalin-corrosive sublimate-salt fluid used by Downey.

**Solution A**

Methylene blue, U. S. P. med. 88 per cent. ....	0.0650 gm.
Methylene azure A 80 per cent. ....	0.0100 gm.
Glycerine, C. P. ....	5.0 c.c.
Methyl alcohol, C. P. ....	5.0 c.c.
Distilled water. ....	25.0 c.c.
Buffer, pH 6.9. ....	15.0 c.c.

**Solution B**

Methylene violet (Bernthsen) 85 per cent. ....	0.0130 gm.
Eosin, yel., W.S. 92 per cent. ....	0.0450 gm.
Glycerine, C. P. ....	5.0 c.c.
Methyl alcohol, C. P. ....	10.0 c.c.
Acetone, C. P. ....	35.0 c.c.

**Buffer**

Primary .....	9.078 gm. $\text{KH}_2\text{PO}_4$ per liter.
Secondary .....	11,876 gm. $\text{Na}_2\text{HPO}_4$ per liter.

The commercial secondary salt has 12 molecules of water and should be exposed to the air for two weeks to bring it down to 2 molecules. To obtain a buffer of about pH 6.9, mix 60 c.c. of secondary salt with 40 c.c. of the primary. The stain solution is prepared by mixing equal parts of solutions A and B. The stock solutions are permanent, but the mixture is good only six or eight months. Kingsley says that the formula may be changed to adapt the stain to the result desired.

*Technique for sections.*

(1) Flood with stain for nine to ten minutes and rinse in clean distilled water.

(2) Wash in distilled water acidified with acetic acid, 15 drops (0.75 c.c.) of 1 per cent solution to 60 c.c. water.

(3) Wash in distilled water and examine under the microscope.

(4) Wipe off excess water and rinse in C. P. acetone to which has been added, for every 60 c.c., 10 drops of 1 per cent acetic acid and also 0.01 c.c. of 5 per cent eosin in  $\text{CH}_3\text{OH}$ .

(5) Rinse in C. P. normal butyl alcohol to which has been added, for every 60 c.c., 0.02 c.c. of 5 per cent eosin in  $\text{CH}_3\text{OH}$ .

(6) Rinse in neutralized xylene. Examine under microscope for proper differentiation.

(7) Three changes of neutralized xylene (change often).

(8) Mount in neutralized xylene-damar.

<sup>25</sup> Kingsley, D. M. *Stain Technology*, 10: 127, 1935.



## E. METHODS FOR FIXED TISSUE LEUCOCYTES

### I. Cells of the Connective Tissue

1. **Preparation of Subcutaneous Tissue Spreads.** Loose connective tissue is found in any vertebrate just under the skin, between that and the muscles. It is usually taken from the abdominal or inguinal regions. After the selected area is shaved, an incision is made in the skin and a very small piece of tissue is snipped off with scissors. The piece is placed on a cover glass and quickly spread out as flat and as thin as possible by means of two teasing needles, taking care, however, not to tear it to the extent of injuring the cells. It is an advantage for two people to work together in doing this, using two needles each. The cover glass is then floated on fixing fluid, the tissue side being down. All the above manipulations must be done quickly before the tissue dries, and it facilitates handling the cover glass if the teasing is done with the cover glass over a black background and supported by a Petri dish which is bottom side up.

2. **Preparation of Omentum and Mesentery Spreads.** There are several ways of taking omentum spreads: on filter paper rings, on test-tube tops, or the cover glass method used for subcutaneous tissue. The object is to get the membranes into the fixing fluid without tearing them or allowing them to dry, and to keep them perfectly flat and uncrinkled. If omentum is to be saved, it is the first thing taken out after the animal is killed. Care should be taken not to expose it to the air unnecessarily.

The filter paper ring method can be used by one operator working alone. A number of rings are cut of fairly heavy filter paper, about 5 mm. to 7 mm. in width and 1 inch in diameter. One of the rings is placed under the omentum so that the tissue is in contact with it all around. Immediately, the ring is cut out and placed in the fixing fluid. The tissue will stick to the filter paper through subsequent operations. After it is stained and taken into xylene, it may be cut from the paper ring as there is then no danger of its becoming wrinkled.

A method of spreading the omentum over the cut-off top of a test tube and tying it there with thread was used by Maximow. For rapid work this requires two operators.

In some animals, such as the frog, the mesentery is a hematopoietic organ. It is easily handled by leaving it attached to the loops of intestine and placing the whole in fixing fluid.

3. **Preparation of Body Wall for Sectioning.** The following method for sections of body wall is taken from Maximow,<sup>25a</sup> 1906.

Shave the skin, make a cut through the body wall and insert a cork frame. Fasten the whole thickness of the body wall to the cork by means of pins if alcohol is used as the fixative, or thorns for a corrosive fluid. Then cut out the cork frame with the tissue pinned to it and carry it through the operations of fixing and hardening. In 80 per cent or 90 per cent alcohol remove the tissue from the cork and trim its edges. Imbed in celloidin, and, in order to get the cells of the subcutaneous connective tissue, section the piece parallel to the surface. This method is suitable for the smaller animals but not for dogs because in them the body wall is too thick. Instead, layers of muscle may be pinned out with connective tissue between them. In fixing body wall in absolute alcohol, Unna states that tannin should be avoided and corks should be soaked in sodium carbonate solution of 2 per cent before use.

4. **Fixation and Stains for Connective Tissue Cells.** For all connective tissue spreads the best general fixation is Zenker-formol used just as described for smears. If mast cell granules are to be preserved, the use of watery fixatives must be avoided and the tissue placed directly in absolute alcohol. Alcohol serves as a quick fixative requiring no washing out, but it makes many cells look vacuolated because of its dissolving action on certain cell constituents. Formalin, also, gives a quick fixation, and for general purposes it can be sufficiently washed out in a few minutes so that the tissue will take a stain. Preparations of subcutaneous tissue and omentum should be stained within a few days after fixation. However, if they have been left for a week or so in alcohol so that some staining capacity is lost, the fixation can be revived by the use of peroxides. The same stains are valuable here as are used on sections.

## II. Cells of the Bone Marrow, Lymph Nodes, and Spleen

1. **Fixation of Wet Smears.** Fixation of the wet smears directly in a liquid is used with such tissues as bone marrow or lymph nodes rather than with blood. For general preservation of leucocytes Maximow's modification of Helly's fluid is the best. Maximow used for this fluid 100 c.c. of Zenker stock solution (without acetic acid) and 10 c.c. of neutralized commercial formalin. Magnesium carbonate may be used for neutralization. Fixation is unusually complete and granules are especially well preserved. It is used according to the following directions:

Float the cover glass on the fixative, smear side down, for fifteen minutes. Do not overfix. Wash in running water for two hours. Place in iodized 70 per cent

<sup>25a</sup> Maximow, A. *Arch. f. mikr. Anat.*, 67: 680, 1906.

alcohol for several hours or overnight. Remove excess iodine by treating with 0.25 per cent sodium thiosulphate solution for about five minutes and washing thoroughly in distilled water. For best results stain within two days. The Helly-Maximow fluid gives especially favorable fixation for hematological stains because of the bichromate it contains.

**2. Fixation for Sectioning.** The usual fixatives may be used, but the time is necessarily longer than for smears and membranes. Zenker-formol, the best fixative, is used for five hours if the piece is of ordinary size, 1 cm.  $\times$  1 cm.  $\times$   $\frac{1}{2}$  cm. Very small pieces are penetrated in three hours. This is followed by twenty-four hours' washing in running water. Formalin is used for twenty-four hours in the strength of 10 c.c. commercial formalin and 90 c.c. of water. After twenty-four hours this should be diluted with an equal volume of water if pieces are to be left in it for a longer time. Bell has found that better results are obtained with old formalin material if dehydration is carried out very slowly, twelve to twenty-four hours in each grade of alcohol. Absolute alcohol is used for pieces of tissue only when watery fluids must be avoided, as in the preservation of mast cell granules.

**3. Bone Marrow Biopsy.** *a. Trephine Method.* Removal of a button of bone by means of a small trephine originated with Seyfarth. A good description of the method of removal is given by Custer.<sup>26</sup> Anesthetize the skin and subcutaneous tissue with 2 per cent novocaine for a distance of 3 cm. above and below the fourth rib. After five minutes, make a 4 cm. sagittal incision in the skin down to the periosteum and fill the subperiosteal space with novocaine. Then cut the ventral table of the sternum with a trephine. Care must be taken not to drill through into the mediastinum. Custer suggests making a mark on the trephine 5 mm. from the cutting edge to show when drilling should stop. The button of bone is broken out by rocking the trephine slightly. Curettings may be made from the marrow cavity. After oozing has ceased from the cavity, the periosteum is replaced and the subcutaneous tissue and skin are sutured.

One-half the bone button should be fixed at once in Zenker-formol, after which it is decalcified and imbedded in paraffin. The other half can be used to make imprints. Imprint preparations are made by taking a small fragment of bone spicules with the marrow adhering and gently touching it to the surface of a clean slip. Imprints can be made and dried more quickly than smears. Dry preparations are best stained in May-Giemsa by the method used for blood films, except that the strength of the diluted stain should be increased. For imprints it may be doubled.

<sup>26</sup> Custer, R. P. *Am. J. M. Sc.*, 185: 617, 1933.

*b. Arinkin Method.*<sup>27</sup> This is the aspiration of marrow fluid through a modified spinal puncture needle. It is simpler than the trephine method and can be repeated on the same patient. It has the disadvantage of not showing cells in situ and of not bringing out cells which lie closely in marrow spaces. Shorten a spinal puncture needle to 3 or 4 cm. Anesthetize the skin, underlying tissue and periosteum at the height of the third or fourth rib and drive the needle through into the spongy bone. When the needle enters the marrow cavity, remove the stilet and attach a syringe. Then about 0.2 c.c. of a mixture of blood and marrow may be drawn out. As much as 10 c.c. can be obtained, but a smaller amount (0.2 c.c.) is better because it is less diluted with blood. Make smears or imprints from this and stain them with May-Giemsa.

**4. Stains.** Delafield's hematoxylin and eosin, the best known tissue stain, shows up general features well in sections of glands or organs especially of human material, but is otherwise little used in a study of leucocytes. Stains which combine an acid dye, such as eosin, with a contrasting basic dye are more valuable. The result obtained can be regulated by varying the pH of the solutions or of the water used for rinsing off the stain. Azure II-eosin and Giemsa are usually diluted with distilled water, but a suitable buffer of the desired hydrogen-ion concentration can be used instead. The stains just mentioned and Dominici, also, sometimes give a result which lacks the pink eosin color. Instead of treating the stains it is often easier to hold the eosin by washing sections before dehydration with 0.2 per cent aqueous acetic acid.

If these stains are to be used on celloidin sections, it is necessary to remove the celloidin before staining.<sup>28</sup>

*a. Azure II-eosin and hematoxylin* as used by Maximow,<sup>29</sup> 1909 and 1924, is a valuable combination which is generally applicable; it can be used on Zenker-formol fixed sections, smears, or spreads, and also on tissue culture preparations. Sections of paraffin or preferably celloidin material are stained first in Delafield's hematoxylin and afterwards in azure II-eosin. The Delafield's stain, not artificially ripened, should be about six weeks old. Make a very dilute solution of it by adding 1 to 2 drops to 100 c.c. of redistilled water. It is as necessary to use pure distilled water here as in working with Wright's blood stain. The solution will be a pale violet color if the Delafield's and the water are both good. Leave sections in the stain for twenty-four hours. Then wash in distilled water for about twenty-four hours. As a result chromatin is blue, and the

<sup>27</sup> Arinkin, M. I. *Fol. Haematol.*, 38: 233, 1929.

<sup>28</sup> Dantschakoff, W. *Ztschr. f. wissenschaft. Mikr.*, 25: 32, 1908.

<sup>29</sup> Maximow, A. *Ztschr. f. wissenschaft. Mikr.*, 26: 177, 1909.

Maximow, A. *J. Infect. Dis.*, 34: 549, 1924.

cytoplasm is either colorless or a light grey. Place in azure II-eosin solution for twelve to twenty-four hours.

*Azure II-eosin solution:*

Stock solution A.

Eosin, water soluble yellowish (w. c.).....	0.5 gm.
Pure distilled water.....	500.0 c.c.

Stock solution B

Azure II.....	0.5 gm.
Pure distilled water.....	500.0 c.c.

Stain

Solution A.....	10 c.c. or 12 c.c. according to material
Solution B.....	10 c.c. or 9 c.c. according to material
Distilled water.....	100 c.c.

Mix the eosin solution and the water first, and then add the azure solution. A noticeable precipitate should not form for several hours. Leave slides upright in the stain for twenty-four hours. Transfer from the stain to 95 per cent or 100 per cent alcohol to differentiate and dehydrate. Finish the dehydration in a second 100 per cent alcohol. Clear in xylene and examine under a microscope. The slides can be further differentiated in 100 per cent alcohol if necessary. Mount in pure, petrol ether-extracted damar, evaporated to dryness and redissolved in xylene C.P.

By this staining combination the chromatin of the nuclei is dark blue, nucleoli are purple and leucocyte granules are differentially stained in colors corresponding to those given by Giemsa. Mast granules may be dissolved away by prolonged treatment in watery fluids. The azure II-eosin solution may be used without hematoxylin. Also, Delafield's hematoxylin may be used in this way preceding such stains as Dominici and Giemsa.<sup>30</sup>

*b. May-Giemsa* is used in a way very similar to that described for bloodfilms: May-Grünwald (diluted) five minutes, Giemsa (1 drop to 1 c.c. distilled water) fifteen minutes. Then wash the section in water, dehydrate in 95 per cent and 100 per cent alcohol and clear in xylene. If the section has taken too blue a tone, the eosin color can be brought out by washing in very dilute acetic acid before dehydrating. Another useful variation is the use of acetone for dehydration and cedar oil followed by xylene for clearing. May-Giemsa is a satisfactory stain only on material which is very well fixed.

The modifications of Giemsa, panchrome and Kardos, are also excellent stains for sections.

*c. Dominici*<sup>31</sup> requires the use of two solutions. First, stain for six or

<sup>30</sup> We wish to express an obligation to Dr. Maximow for checking this description.

<sup>31</sup> Dominici, *M. Compt. rend. Soc. de biol.*, 54: 221, 1902.

seven minutes in an eosin-orange G solution (eosin  $\frac{1}{2}$  gm., orange G  $\frac{1}{2}$  gm., and distilled water 100 c.c.). Rinse off the excess stain in distilled water but do not leave the section in water long enough to fade. Then stain in 0.5 per cent aqueous toluidine blue solution for twenty to thirty seconds. Rinse in distilled water and differentiate in 95 per cent alcohol, controlling the result by a rapid glance at the preparation under a microscope. Finish the dehydration with 100 per cent alcohol and clear in xylene. Eosin B. A. of the Hollborn dyes and yellowish eosin of the American dyes are good in this stain.

Variations can be made by using acid fuchsin (0.5 gm.) in place of eosin, or azure A (0.1 per cent aqueous) in place of toluidine blue.

Another variation is the use of phloxine-orange G, suggested by Conn. It is recommended especially for material on which a brighter pink than eosin is desired, or for formalin material on which eosin-orange G does not take. Suggested proportions are phloxine 0.12 gm., orange G 0.30 gm. and distilled water 100 c.c. Use with toluidine blue as above.

*d. Phloxine-azure* (Haynes)<sup>32</sup> is a modification of Mallory's phloxine-methylene blue. Use after Bouin or Zenker fixation. Stain in 2.5 per cent aqueous phloxine solution for fifteen minutes. Wash in water and stain in 0.1 per cent aqueous solution of azure A for thirty minutes. Wash in water and differentiate in 95 per cent alcohol with a few drops of xylene-colophonium. Finish the dehydration with absolute alcohol and clear in xylene.

*e. Methyl green-pyronin* (Pappenheim)<sup>33</sup> is particularly brilliant on sections of spleen and lymph nodes because it gives a bright red color to the cytoplasm of large lymphocytes and plasma cells. It is especially valuable when it is desired to distinguish between chromatin and other basophilic materials in the cell, as the method is specific for chromatin. The latter is stained a dark, greenish-violet color, while other basophilic constituents of the cell are colored red with the pyronin of the mixture. The nucleolus is red if of basic staining reaction and colorless if acid. The method is, therefore, a very convenient one for distinguishing between chromatin and nucleolar material. It should be used after Zenker-formol or alcohol fixation. It cannot be used after formalin alone. Grawitz formula follows:

Solution A

Phenol crystals, liquefied.....	0.25 gm.
Distilled water.....	100.00 gm.
Methyl green.....	1.00 gm.

<sup>32</sup> Haynes, R. *Stain Technology*, 1: 68, 1926.

<sup>33</sup> Pappenheim, A. *Virchows Arch.*, 157: 19, 1899.

## Solution B

Phenol crystals, liquefied.....	0.25 gm.
Distilled water.....	100.00 gm.
Pyronin.....	1.00 gm.

## Stain

Solution A.....	15 parts
Solution B.....	35 parts

After several weeks or months a precipitate may form in the staining solution. Stain for about six minutes. Rinse briefly in distilled water, dehydrate in acetone, and clear in cedar oil followed by xylene.

The formula already given is adapted to Grüber dyes. Modern pyronins have more staining strength so that the amount of methyl green should be increased in proportion. Pyronin Y of the National Aniline and Chemical Company gives satisfactory results when made up by the Unna-Pappenheim formula, methyl green being added. Unna-Pappenheim formula with 0.35 gm. of extra methyl green:

Methyl green.....	0.50 gm.
Pyronin Y.....	0.25 gm.
Alcohol.....	2.50 c.c.
Glycerine.....	20.00 c.c.
0.5% carbolized water to.....	100.00 c.c.

Stain five minutes or more. The time should be carefully adjusted as one minute makes a difference.

f. *Alcoholic thionin* (50 per cent alcohol saturated with thionin) is used on material which has been fixed in absolute alcohol for the preservation of mast cells. Stain for twenty-four to forty-eight hours in a covered dish. A solution which acts more quickly can be made by adding 4 drops of 2 per cent  $\text{Na}_2\text{CO}_3$  to 20 c.c. of the thionin solution and leaving it overnight. The solution should be filtered before use. Stain for ten to twenty minutes. Thionin is a metachromatic stain and colors nuclei blue and mast cell granules a brownish-red to violet or purple color.

g. *Indulin-aurantia-eosin* (Ehrlich)<sup>34</sup> and Ehrlich's triacid-toluidine blue have their greatest usefulness on marrow smears and similar material fixed in Zenker-formol.

The indulin-eosin formula is as follows:

Indulin.....	2 gm.
Aurantia.....	2 gm.
Eosin.....	2 gm.
Glycerin, pure.....	30 gm.

Dissolve the mixture in the oven at about 40°C. Keep the stain well stoppered.

<sup>34</sup> Krause, R. *Enzyklopädie der Mikroskopischen Technik*, Berlin, 1926, p. 1049.

To use, cover a smear with stain and place it in an oven at 40°C. for four to five and one-half hours. Rinse in distilled water. Dehydrate in 95 per cent and 100 per cent alcohol. Clear in xylene. This stain gives an excellent differential result on granules. Preparations keep well.

*h. Ehrlich's triacid-toluidine blue* is one of the most brilliant stains for bone marrow cells. According to our experience, it fades in a year or two. The triacid stain is discussed in connection with dry blood films. For smears fixed in Zenker-formol the following procedure is used: Place the smear in triacid for five minutes, rinse briefly in distilled water and stain in 0.5 per cent aqueous toluidine blue for ten seconds. Then rinse again in water, dehydrate beginning with 95 per cent alcohol and clear in xylene.



# HISTOLOGICAL TECHNIQUE FOR THE STUDY OF BONE

P. G. SHIPLEY

General considerations 344. Preparation of sections of whole bone without decalcification 345. Preparation of bone from which organic matter has been removed 345. Decalcification 346. Isolation of bone corpuscles 348. Isolation of lamellae 348. Staining 348. Demonstration of calcified tissue in bone 349. Demonstration of lacunae and canaliculi 349. Demonstration of processes of young osteoblasts in growing area of bones 351.

## I. GENERAL CONSIDERATIONS

Histological methods which have been devised especially for the study of bone, in general have to do with the removal from this tissue of the lime salts with which it is impregnated, and which, unless they are removed, make it impossible to cut the bone into sections for study. These methods depend for their efficiency on the solubility of the tertiary phosphate and the carbonate of calcium in strongly acid solutions and are equally efficacious, and satisfactory in that they remove the inorganic material from the bones and leave the tissue softened and in a proper state for cutting. The majority, however, fail because the strongly acid solutions so sear the soft tissues to the calcified structures, and cause so much destruction and shrinkage as to make the normal structure and relations of the cellular tissue highly conjectural. In order to obviate this difficulty many authors have suggested the use of weak organic acids. While decalcification with the latter allows the preparation of much better sections, both of the calcified and soft tissues, it is extremely time consuming, and requires a great deal of patience in application. The good results obtained by the use of these weak decalcifying agents, nevertheless, are worth the time and patience required.

It must not be forgotten in this connection that certain reagents used for fixation or hardening soft tissue, will, if their action is long enough continued, decalcify even quite large calcareous bodies. This action is dependent on the presence or evolution in them of small amounts of one acid or another, and may be abolished by neutralization.

The *calcified part* of bone is studied by methods which have been devised for making it visible after destruction of the organic adnexa. Moreover, certain special ways have been worked out for the easy demonstration of some of the structures peculiar to osseous tissue.

Bits of bone which are sufficiently small and included in soft tissue

may be cut after being imbedded in celloidin but they will invariably destroy the edge of the knife, which will require constant sharpening.

## II. PREPARATION OF SECTIONS OF WHOLE BONE WITHOUT DECALCIFICATION

The following method was worked out by Grieves for the study of dental tissues and is eminently satisfactory for preparing sections of bone with the soft tissues attached:

1. Small bones or pieces of bone with marrow or other soft tissues are fixed for twenty-four to thirty-six hours in formalin 10 per cent or other desired fixing agent.
2. Wash in running water twenty-four hours, then one hour each in 2 changes of distilled water.
3. Dehydrate in graded alcohols beginning at 50 per cent.
4. Treat with absolute alcohol and chloroform, equal parts, two hours.
5. Follow with chloroform two hours.
6. Pass the tissue into solutions of window glass rosin<sup>1</sup> in chloroform as follows: Two hours each in 5 per cent and 10 per cent solution, then into a saturated solution until it becomes transparent. Finally the tissue is imbedded in rosin in the following manner:

Three small glass dishes containing melted rosin are maintained in that condition on a copper bar, and the bone is passed through them, remaining one minute in each. Evaporation of any chloroform remaining from the final solution of rosin in chloroform occurs during this process. The block is then allowed to cool and is ground as thin as possible by hand on carborundum stones. One surface having been polished on a fine hone, it is fastened to a warm glass slip with a bit of melted rosin, care being taken to exclude air bubbles between the section and the slip. It is then rubbed down to the desired thinness. The remaining surface having been polished on a very fine hone or on glass, the section is ready to be treated by the method of staining described below. All grinding must be done under lukewarm water.

## III. PREPARATIONS OF BONE FROM WHICH THE ORGANIC MATTER HAS BEEN REMOVED

These are made by digestion in alkalis, or by tryptic digestion, by calcination, by drying in air or in sand, through the agency of insects as by exposing the tissue to ants, by bacterial action involving putrefaction and by autolysis. Perhaps the best method for accomplishing this result is to trim and scrape all the soft tissue away from the bone which may or may not be split. The bone is then placed in tap water or in a weak

<sup>1</sup> Window glass or water white "rosin" (not resin) is a very pure transparent pine rosin used in dental technique and may be obtained from dealers in dental supplies.

(2 per cent) solution of gelatin to which a loopful of a culture of *B. coli* has been added. In about five or six days the bone is removed from the culture and thoroughly rinsed in running water for twenty-four to forty-eight hours. All the organic matter will, as the result of this procedure, have been dissolved and washed away. The whole process should of course be carried out in an unfrequented and well-ventilated place as the odors evolved during putrefaction are intolerable. It is as well to sterilize bones so prepared, by boiling, or by immersion in alcohol before going further with their treatment. When the bone has dried completely it may be sawn into sections, which may be ground to the requisite degree of thinness either by hand or on such an apparatus as Coriel's laps or on Black's instrument. The finished section should be polished on glass or a fine hone and after dehydration in ether should be mounted, when thoroughly dry, in thick balsam and covered.

#### IV. DECALCIFICATION

1. **With Solutions of Inorganic Acids.** Hydrochloric acid has long been used for decalcification in 1 to 2 per cent and 3 per cent watery and alcoholic solutions and in 5 per cent solution in glycerin. Its action is very rapid but it is not to be recommended as it swells the tissues greatly and I do not feel that such substances as salt, chromic acid or alcohol have any effect in preventing the results of its deficiencies. I have had no experience with mixtures with palladium chloride.

a. *Nitric Acid.* Like hydrochloric acid, nitric acid can be recommended for use only when very large masses of bone are to be decalcified very rapidly. It may be used in strengths varying from 1 to 10 per cent in either alcoholic (70 per cent or 95 per cent) or watery solution which should be changed every day. After decalcification the tissue should be washed in running water and transferred to 95 per cent alcohol, which should be changed three times. It is better to bring the tissue into 95 per cent alcohol before putting it into the decalcifying fluid.

b. *Sulphuric acid* is perhaps the poorest decalcifying agent.

c. *Sulphurous acid* is used in saturated solution after fixation in formalin. It is said to preserve tissue well.

d. *Formic acid* for softening large masses of bone is to be preferred to any other. It may be used in water solution but is best at a 1 to 5 per cent strength in 70 per cent alcohol. Tissues should be fixed in formalin and after decalcification must be washed in 70 per cent alcohol, not water. Decalcification in 5 per cent solution goes to completion in four to five days even when large masses of bone are used.

2. **With Weak Acids.** After extensive experimentation with many methods of decalcifying bone the writer has come to rely on prolonged

immersion in Müller's fluid as the best and most reliable means. The decalcification is effected through the liberation of small amounts of chromic acid from the potassium bichromate in the solution. Bones are sawn or cut into pieces or split and allowed to remain in formalin (10 per cent solution) for seventy-two hours and then immersed in Müller's fluid and kept in it until they bend or can be pierced easily with a needle. The fluid should be changed occasionally but not too frequently, not oftener than once in two weeks, since more frequent changing slows the decalcification very much. Decalcification by this method is very slow but the results obtained through its use fully compensate for the delay. Removal of the lime from the bones of a normal adult rat occupies from twenty-one to thirty days in Müller's fluid. The process may be hastened somewhat by being carried out in an incubator at 37°C. Over-decalcification is practically impossible in this fluid. This is the only decalcifying agent which allows the determination of the amount of osteoid tissue present in the bone during life, since all lamellar substance which was calcified is basophilic and stains a more or less intense blue with hematoxylin. Osteoid tissue alone is acidophilic. Soft tissues are beautifully preserved. This fluid may be followed by almost any stain including those used for coloring mitochondria.

*a. Chromic acid* is often recommended alone and in mixtures with other acids as a decalcifying agent, e. g., equal parts of 3 per cent chromic acid and 1 per cent hydrochloric acid. Solutions containing chromic acid alone are very weak agents and cause much shrinkage. One-half per cent is the best solution to use. Mixed with other acids chromic acid fails to prevent undesirable effects. The following acids are used as indicated:

*b. Picric acid* in saturated solution is very weak but can be used for small objects.

*c. Phosphoric acid* is used in 10 per cent to 15 per cent solution. This acid ruins staining possibilities in most tissues. For very small bones it may be taken in solutions of 0.5 per cent.

*d. Lactic acid* in solutions of 10 per cent strength is recommended by some workers. This acid causes much swelling.

*e. Arsenic acid.* 5 per cent is the strength recommended in which this acid should be used. I have no experience with results from decalcification by this substance.

*f. Trichloroacetic acid* decalcifies in 4 to 5 per cent solution.

### 3. With Phloroglucin.

Phloroglucin is always used in combination with acids such as hydrochloric, nitric, or sulphuric. It is not a decalcifying reagent but protects soft tissues against the strong acids and allows their use in high concentration. A saturated

solution of phloroglucin is made, and to it are added 5 to 40 per cent of nitric acid. After decalcification the bone may be washed in running water. Another good formula is the following:

Nitric acid.....	5.0 c.c.
Phloroglucin.....	70.0 c.c.
Alcohol.....	1.0 gm.
Distilled water.....	30.0 c.c.

The latter decalcifies somewhat more slowly.

#### 4. With Magnesium Citrate.

Kramer and the author have evolved a method of decalcification in neutral solutions which is very satisfactory for small pieces of material which have been fixed in formalin. This method depends on the solubility of tertiary calcium phosphate in water in the presence of citrate of magnesia. The reagent is prepared as follows: 80 gm. of citric acid are dissolved in 100 c.c. of hot water; 4 gm. of magnesium oxide are added and the mixture is stirred until a complete solution is made. Cool and add 100 c.c. of ammonium hydroxide (density 0.90) and dilute to 300 c.c. Allow to stand twenty-four hours and filter. (If the magnesium oxide contains much carbonate it should be freshly ignited.) Titrate with 5N/hydrochloric acid to approximately pH 7.0—7.6 and add an equal volume of distilled water. This solution should be changed every three days. It decalcifies with moderate rapidity (fifteen days for a dog's rib) and preserves the architecture of soft tissue very well. Best results are obtained by using large quantities of the solution. Ammonium citrate has been used for the same purpose.

### V. ISOLATION OF BONE CORPUSCLES

This is easily carried out as follows: A thin section of bone is put into concentrated nitric acid for from a few hours to a day. The section is then placed on a slip with a cover glass over it. Pressure on the cover will squeeze out the ellipsoid bone cells with their processes.

### VI. ISOLATION OF LAMELLAE

This may be accomplished by decalcifying bones and then allowing them to simmer in water. The lamellae will peel off readily after this treatment, often by themselves.

### VII. STAINING

To prepare bone, there would seem to me to be no better fixative than a 10 per cent solution of formalin. Almost any method which can be applied to any other material may be used to color sections of osseous

tissue. For ordinary purposes the writer knows of none which give better results than hematoxylin and eosin, although some prefer carmine as a contrast stain. Iron hematoxylin and phosphomolybdc hematoxylin give very beautiful preparations. Celloidin is much to be preferred to paraffin as an imbedding matrix.

### VIII. DEMONSTRATION OF CALCIFIED TISSUE IN BONE

To demonstrate the calcified tissue in bone, special stains for osseous tissue are used. The lamellar tissue which was impregnated with lime during life is basophilic after decalcification in Müller's fluid or magnesium citrate and is colored blue by hematoxylin, blue-black by phosphomolybdc and black by iron hematoxylin, etc., but the most convenient method of demonstrating the extent of calcification is that of v. Kossa. This depends on the formation and reduction of silver phosphate or carbonate and the precipitation of metallic silver under the influence of light. It may be carried out with frozen or celloidin sections or with the surfaces of blocks of fresh bone. Material as described above is immersed in a 1 per cent solution of silver nitrate, after washing in distilled water to clean the tissue of blood or debris. It is then exposed to the full light of an arc or tungsten filament in the nitrate solution and allowed to remain until no further blackening occurs. It should be removed before the soft tissues begin to turn brown. This staining does not penetrate deeply into the bone. The tissues must not have been exposed to acids before immersion lest some of the lime salts be removed, but they may be decalcified after the precipitation of the silver since the latter metal remains in situ in spite of the removal of the calcium. Preparations made by v. Kossa's method are not permanent, but they may be kept a few months if they are well washed in distilled water and treated with sodium thiosulphate to remove the excess of silver salts after impregnation.

### IX. DEMONSTRATION OF THE LACUNAE AND CANALICULI

The simplest method of demonstrating the lacunae and the canaliculi of dried bone is as follows: 1. Sections of bone ground to required thinness are placed in 0.75 per cent silver nitrate solution and allowed to remain twenty-four hours. After washing the section, polish it on a fine hone to remove the precipitated silver, dehydrate in alcohol and imbed in balsam from xylol. Lacunae and canaliculi are black or yellowish brown.

1. **Impregnation with Acid Fuchsin.** Thin sections of dried bone

are extracted with alcohol and dried thoroughly. They are then put in watch crystals containing a 20 per cent solution of acid fuchsin. The dishes are placed in a desiccator which can be connected to a suction apparatus. The air is extracted for about an hour and the desiccator locked. After twenty-four hours the solution will have dried. The sections should be removed and the superfluous precipitate rubbed off on a fine carborundum stone. They should then be polished on a hone and passed through xylol to be mounted in damar or balsam.

**2. Methods for Staining Linings of Lacunae and Canaliculi.** The linings of the lacunae and canaliculi can also be stained beautifully by the following:

*a. Krause's Method for Frozen Sections.*

- (1) Gram's solution, 25 c.c. Water, 25 c.c., one-half hour.
  - (2) Wash.
  - (3) Gold chloride 2 per cent solution until the sections are yellow.
  - (4) Wash and reduce in a 2 per cent solution of resorcin, one to two hours.
  - (5) Wash.
  - (6) 5 per cent solution of hyposulphite of soda, fifteen to thirty minutes.
- This method is recommended by Krause for frozen sections.

Of the two staining methods described by Schmorl the writer has found the second by far the more satisfactory. The methods are given as the writer has used them:

*b. Sections in Celloidin (Modified Schmorl Methods).*

*First Method.*

- (1) Fix in formalin.
- (2) Decalcify in Müller's fluid.
- (3) Wash, dehydrate and imbed in celloidin.
- (4) Wash in water thirty minutes.
- (5) Stain in the following solution for ten minutes:
 

Saturated solution of thionin in 50 per cent alcohol.....	2 c.c.
Water.....	10 c.c.
- (6) Wash.
- (7) Decolorize (one-half to one minute in a saturated solution of picric acid).
- (8) Wash.
- (9) Treat with 70 per cent alcohol until the color ceases to come off rapidly.
- (10) Dehydrate in 95 per cent alcohol.
- (11) Clear in oil of origanum.
- (12) Mount in damar.

*Second Method.*

- (1) Fix in any fluid not containing mercury.
- (2) Decalcify in Müller's fluid.
- (3) Wash in running water.

- (4) Dehydrate, imbed in celloidin, cut sections not over  $10\mu$ .
  - (5) Stain in thionin solution made alkaline with 2 drops of ammonia.
  - (6) Transfer with a glass needle to a saturated solution of phosphotungstic or phosphomolybdic acid until the sections are blue or gray or green.
  - (7) Place in water until sections are sky-blue.
  - (8) Transfer into the following solution for three to five minutes:
 

Ammonium hydroxide .....	1.0 c.c.
Water .....	10.0 c.c.
  - (9) Pass through several changes of 90 per cent alcohol and into
  - (10) 95 per cent alcohol.
  - (11) Clear in carbol xylol.
  - (12) Mount in damar.
- This method is recommended for the bones of children.

#### X. DEMONSTRATION OF THE PROCESSES OF YOUNG OSTEO-BLASTS IN THE GROWING AREA OF BONES

Thin slices of bone from a rickety animal are cut and treated in the manner following:

- (1) Place in a 4 per cent solution of citric acid in distilled water for twenty to thirty minutes in the dark.
- (2) Rinse in distilled water.
- (3) Transfer to a 1 per cent solution of gold chloride in distilled water in the dark for twenty to thirty minutes.
- (4) Place in a 33 per cent solution of formic acid in the dark for forty-eight hours.
- (5) Rinse in distilled water.
- (6) Preserve in pure glycerin.

Frozen sections should be made and mounted in glycerin. Ring the cover with damar, balsam, paraffin or cement. When not in use the slides should be kept in the dark.

**1. Gries' Stain for Sections of Hard and Soft Tissues.** The slide prepared as described above is rinsed in distilled water and treated as follows:

- (1) 20 per cent alcohol fifteen minutes.
- (2) Stained in cresylechtviolet solution twelve to twenty-four hours according to the thickness of the section.
  - (a) Stock solution of cresylechtviolet..... 1 part
  - (b) Distilled water..... 99 parts

The stock solution of the dyestuff is made as follows:

Cresylechtviolet .....	1 gm.
Phenol (5 per cent solution) .....	50 c.c.
Alcohol 95 per cent .....	20 c.c.



The dye should be thoroughly suspended in the phenol before the alcohol is added.

- (3) Rinse in distilled water.
- (4) Differentiate in acetone.
- (5) Clear in bergamot oil and creosote.
- (6) Mount in damar.

The acetone differentiates the stain, dehydrates the section and dissolves the rosin with which it was impregnated.

## TECHNIQUE FOR THE STUDY OF DENTAL TISSUES

H. R. CHURCHILL AND J. T. L. APPLETON

By the "dental tissues" we mean: (1) the enamel cuticle or Nasmyth's membrane; (2) the enamel; (3) the dentine; (4) the pulp; (5) the cementum; (6) the dental periosteum or periodontium; (7) the epithelial attachment; (8) the marginal aspect of the gingiva and (9) the alveolar bone. The structures 5-9 form a functional unit whose task is to resist the physiological forces applied to the tooth. It is by means of the latter tissues that a tooth is attached to the jaw. Collectively they are called the attachment apparatus or parodontium.

The chemical composition, the physical nature and the structural arrangement of the dental tissues present difficulties in their histological examination which are met in various ways.

Many structural details of enamel, dentine, cementum and alveolar bone can be identified in sections prepared by grinding.

To study the relations of the hard structures to the pulp or those of the tooth proper to its attachment apparatus it is necessary to use some decalcifying method followed by microtome sectioning or some method of petrification followed by grinding.

### A. EXAMINATION OF THE TOOTH AS A WHOLE

1. **Simple Grinding.** If it be desired to preserve the enamel, the specimen should at no time be allowed to become dry. Drop the tooth into a neutral physiologic sodium chloride solution with 4 per cent formalin, immediately after removal from the body. If the grinding is to be done within a few hours, the physiologic solution alone will do. Divide the tooth in the plane of the section by cutting it with a fine saw while it is held in a vise between two pieces of cork.

The grinding can be done in three ways: 1, by hand on a flat carborundum stone; 2, on a carborundum wheel driven by a motor; or 3, between two plates of frosted glass. The combination carborundum stone No. 108 (5 cm.  $\times$  120.4 cm.) is very satisfactory for hand grinding. On one side is grit No. 120, on the other 2 f. The coarser grit should be used only long enough to get the two sides of the tooth parallel, then the grinding should be continued on the finer side. The surface should be constantly flooded with water. A good way is to lay the stone in a glass photographic developing tray of sufficient size, with a blotter on the bottom, to keep the stone from slipping. Water can be kept in the tray

almost to the level of the upper surface of the stone. From this as a reservoir the surface can be frequently flooded. When the section has almost reached the desired thickness, it should be transferred to a very fine stone (carborundum, "60 minute" grit or an Arkansas stone) for final polishing. Polishing can also be done on plate glass with a thin paste of the finest precipitated chalk. The specimen, while it is being moved back and forth, is conveniently held by a cork, in size large enough to cover the specimen and about 10 to 15 mm. high, which permits the fingers easily to take hold. The grinding should never be so fast as to cause cracking. The optimum rate is learned only by practice. If one side of the tooth is being ground down more quickly than the other, this can be corrected by altering the direction of pressure on the cork.

When the polishing is completed, transfer the section by means of a camel's hair brush and section lifter to a dish of clean distilled water, and there thoroughly free it from the debris of grinding. Several changes of water are necessary. Then pass the section through the alcohols, into xylol and mount in balsam. It is often well to hold the cover glass down gently with a wire spring until the balsam has hardened.

The interglobular spaces in the dentine and the branchings of the dentinal tubules will be rendered more conspicuous if they contain air before the balsam is added. Consequently for this purpose remove the section, after immersion in 95 per cent alcohol, to the slip, and blot firmly against it. If the air of the laboratory be dry, exposure for an hour or two, before putting on the balsam and cover glass, will suffice. In this case the wire spring to hold the cover glass in place will be almost indispensable, because on drying, the section, if very thin, usually becomes warped, which is highly undesirable for microscopic examination. It should be remembered that cracks usually appear, particularly in the enamel, when the section is allowed to become dry.

**2. Grinding against a Revolving Wheel-stone.** The grinding can be greatly accelerated by the use of motor-driven, wheel-shaped stones with a coarse and a medium grit wheel. The position of the stone may be either vertical or horizontal. The grinding is done against the flat surface of the wheel, which should, before use, be "trued" by holding against it while rapidly rotating a "trued" flat carborundum stone of the same grit. A constant flow of water should be kept over the surfaces in contact. The stone can be kept moist at this time and for the grinding of the tooth by means of a simple system, consisting of a small, fine-pored sponge lightly held in contact with the revolving stone and supplied with water through a small-bored tube. The flow of water can be regulated by means of a screw pinch-cock when the connecting tube is of rubber. The "trueing" of the carborundum wheel need be done only when first purchased.

The coarser stone should be used only until the surfaces of the tooth are parallel. Most of the grinding will be done with the medium stone. At first it will be easier to hold the specimen with the fingers, but when the surfaces are once parallel, holding by means of the cork can be substituted. During the grinding the tooth should be constantly moved back and forth along the line of a radius of the stone. The speed of rotation should not be great and the specimen should not be pressed tightly against the cutting surface. It is of advantage to be able to increase the speed of the motor gradually.

The final polishing must be done by hand on a very fine stone.

Sections of the hard dental tissues may be ground between two plates of ground or frosted glass. The larger, *ca.* 8 inches square, is placed, smooth side down, on a wet towel or a wet piece of newspaper, one layer thick, to keep it from sliding. On the upper surface is placed a pinch of finely powdered glass or pumice stone or carborundum powder with plenty of water, and one surface of the specimen is ground flat. The specimen is held in the fingers and a broad, rotary motion is used. When one surface has been ground flat, the specimen is turned over and the other surface is flattened. The specimen must be held in the fingers until the section is 0.5 mm. or less thick. Then the upper square of frosted glass (*ca.* 4 inches square), smooth side up, is put over the specimen and water run in from the side so that almost the whole space between the two pieces of glass is filled with water. The abradant should be used sparingly. The upper plate, held with the fingers, is then moved with a broad, not too rapid rotary motion. The specimen is easily seen through the upper piece of glass. Grinding is continued until the section almost falls apart. When this is likely to happen can only be learned by experience. Considerable practice should be acquired, in this method as well as in any other grinding method, before one begins to work with important or valuable specimens. The section is removed carefully for final polishing on a very fine stone, by hand.

Hand ginding is tedious and grinding against a motor-driven wheel as just described is almost as bad. Consequently several machines have been devised to make the operation as automatic as possible.<sup>1</sup>

The principle underlying the construction of these machines is the same. The motor-driven grinding stone is fixed in a vertical position at one end of a lathe.

<sup>1</sup> Allen, F. W. *Dental Cosmos*, 45: 27, 1903.

Black, G. V. In: Noyes, F. B. *Dental Histology and Embryology*. Ed. 2, Phila., 1915. Appendix Chap. 1, pp. 381-401.

Patten, B. M., and Chase, S. W. *Anat. Rec.*, 30: 123, 1925.

Boedeker, C. F. *Fundamentals of Dental Histology and Embryology*, N. Y., 1926, p. 228.

The carrier is affixed to the end of a horizontal shaft, held without play in a casing which slides on the lathe. This shaft can move freely to or from the stone. The other end of the shaft connects with a spiral steel spring. This maintains a constant but slight pressure of the specimen against the grinding surface. The spring can be set to vary the pressure as desired. A set-stop, calibrated to represent the thickness of section in micra, can be adjusted so that when the desired thickness is reached the spring no longer acts and grinding stops. The section is then removed from the carrier by some balsam-solvent, polished by hand, and mounted as desired.

In these machines, the lathe should be heavy in order to minimize vibration and its parts should permit as little play as possible. An abundant supply of cold, preferably iced, water should flow over the stone and the specimen.

The specimen is prepared for the grinding process as follows: One side of the specimen is ground flat and polished. This side is cemented by balsam to a brass section-holder. Several sections may be so cemented at one time. The "proper" consistency of the balsam is of the utmost importance. It should be just thick enough to become solid, but not brittle, in ice water. If the balsam is too thin, the slice will "creep" on the surface of the object disc during grinding. If it is too thick it becomes brittle and the slice breaks from the object disc. The most desirable consistency seems to vary considerably in different lots of balsam and can be determined only through personal experience. Black stated that it should be stiff enough to move sluggishly at 110°F., but fluid at 120°F. or 130°F.<sup>2</sup>

For the purpose of cementing the specimen to the section holder, Chase<sup>3</sup> has used an adhesive cement composed of dissolved celluloid in place of Canada balsam, and finds it much more satisfactory. The cement may be made by simply dissolving clear celluloid in acetone until the consistency is that of table syrup, or a better and more convenient cement may be bought in tubes under the name of Dupont's Household Cement. This adhesive has several advantages over balsam: it is easily and inexpensively procured and kept; it is uniform in quality; specimens affixed by it may be ground in the presence of water at room temperature; it does not fill and clog the surface of the wheel or bone as rapidly as does balsam; one can be practically certain that the specimen will remain attached to the mount throughout the grinding process, if the cement is properly used. The following technique for the use of "Household Cement" is employed in his laboratory:

<sup>2</sup> Patten, B. M., and Chase, S. W. *Anat. Rec.*, 30: 123, 1925.

<sup>3</sup> Chase, S. W. *J. Dental Research*, 10: No. 3, 1930.

- (1) Fix the specimen or not as desired.
- (2) Grind and polish one face of the specimen at the desired plane of section.
- (3) Clean the polished surface with water, rubbing gently with a cloth free from lint.
- (4) Dehydrate the specimen: twenty to sixty minutes each in 35, 50, 70 and 95 per cent alcohols.
- (5) Acetone: twenty to sixty minutes.
- (6) Apply a thin film of Dupont's Household Cement to the clean surface of the grinding mount.
- (7) Rapidly apply a similar film to the polished surface of the specimen.
- (8) Quickly press the specimen on the mount, using a slight rotary motion to ensure uniform union of the two films.
- (9) Immediately plunge the mounted specimen into chloroform where it should remain for at least thirty minutes.
- (10) Grind and polish in the constant presence of water.
- (11) Transfer polished thin section on mount to 95 per cent alcohol five to ten minutes.
- (12) Free the section from the mount by soaking in acetone. Wipe gently to clean.
- (13) Place the freed section in xylol for five to ten minutes.
- (14) Mount in Canada balsam.

Note: Stains or other treatment may be applied between steps 10 and 12 or between 3 and 5.

An objection to the grinding method as outlined above is that only one or two sections in parallel planes can be prepared from a tooth; however, several longitudinal sections can be obtained from one specimen approximating the human tooth in size, by the use of various slicing machines devised for this purpose. Black<sup>4</sup> recommended a motor-driven aluminum disc (copper is frequently used) (24 to 30 gauge and about  $3\frac{1}{2}$  inches in diameter). The tooth to be sliced is fixed in a brass tube slotted at the free end, with plaster of Paris or sealing wax. This tube is clamped into an object holder fixed upon the slide rest of the lathe. The object holder may be swung horizontally to any possible position in relation to the aluminum disc. When set, the object is moved slowly against the revolving disc, which is fed with carborundum powder suspended in soapy water.

The following technique for the rapid preparation of ground sections of individual teeth has been used by Johnston of the Dental Study Group at Yale University.

After extraction the teeth are fixed in formalin until ready for cutting. The teeth are dried and affixed to the end of a wooden block by

<sup>4</sup>Black, G. V. In: Noyes, F. B. *Dental Histology and Embryology*. Ed. 2, Phila., 1915. Appendix Chap. I, pp. 381-401.

modelling compound. The block is placed and firmly fastened in the tool holder of a power lathe.<sup>5</sup>

Sections cut from the tooth by cutting wheels<sup>6</sup> which are 4 inches in diameter and 0.01 to 0.02 inch in thickness. They have a 0.5 inch center hole for application to the drive shaft of the lathe. For a single section two wheels are used with a 0.01 inch space washer between them. Because of the thinness of the cutting wheels they are supported on the outside by steel washers. Three cutting wheels each spaced by washers, which determine the thickness of the section cut, will furnish two sections from the same tooth by the same operation. A multiplicity of sections may be obtained by the use of a continued series of cutting wheels over the width of the tooth.

The tooth mounted on the wooden block is advanced on a screw drive, regulated by hand toward the cutting wheels, which are bathed by a constant stream of water. High speeds of the drive shaft should be maintained and only sufficient pressure is placed on the advancing tooth to contact the cutting wheel.

The foregoing technique produces a smooth, polished "ground section" in much less time than was previously consumed by the older grinding methods.

Occasionally it is necessary to use a Belgian stone to smooth and thin the section. The sections are dehydrated in the usual manner with various grades of alcohol, cleared in xylol and mounted in balsam.

**3. Petrification Method.** The methods of grinding sections so far considered permit only the examination of the hard substances of the tooth. It is obviously important for some purposes to have a method which will preserve the hard and soft dental tissues in their natural relations. In most instances this is accomplished by decalcification which, however, destroys in toto mature enamel. When it is desired to preserve all the dental tissues including enamel in one section, the only method available is that of petrification. This is usually known as the Koch-Weil<sup>7</sup> process, of which several modifications have been described.

The object is cut by a saw or a slicing machine into sections as thin as the technician believes it to be possible without distorting the tissues. These slices are then fixed and stained in bulk. Grenachler's alcoholic

<sup>5</sup> Any stationary motor may be adapted for a drive shaft and a feed apparatus can be easily constructed.

<sup>6</sup> Cutting wheels have been obtained from the H. Allison Co., Bridgeport, Conn.

<sup>7</sup> S. W. Chase (*J. Dental Research*, 10: No. 3, 1930) stated: "Weil's ('87) more rapid evaporation of the balsam solvent was not an improvement of Koch's method, as Röse ('92) has pointed out, and does not justify the usual practice of calling the method the Koch-Weil method. Christensen erred in ascribing the method to Weil (*Dental Cosmos*, 36: 284-290, 1894)."

borax-carminé for a fortnight or aniline blue-black have been recommended.<sup>8</sup> After staining, dehydration is accomplished by gradually increasing strengths of alcohol. After removal from absolute alcohol, the pieces are cleared in oil of cloves or oil of cedar wood for twelve hours. The oil is quickly washed off with xylol and the pieces are suspended in pure chloroform for twenty-four hours. Then they are put in chloroform balsam for a day, when more dry balsam is added until the chloroform can take up no more. This occupies three to four days. Finally the container (an individual container for each specimen, made of heavy lead foil, is very useful—Boedeker) with the pieces is put over a water-bath whose temperature should not rise above 90°C. A drying oven maintained at this temperature would be better. In two to three days (it may take much longer) when the thoroughly cooled balsam cracks like glass on the introduction of a needle point, the slices are removed and ground to the desired thickness by any of the grinding methods outlined above. The impregnation with balsam can be hastened if it be carried out in a partial vacuum chamber.

A modification of this method, using celluloid in place of balsam, was introduced by Chase<sup>9</sup> who pointed out the following advantages:

- (a) It is more rapid, requiring only days instead of weeks (though, as Röse (1892) has shown, too much celerity is undesirable).
- (b) It is not necessary to grind the specimen in the presence of ice water.
- (c) The celluloid does not clog the stones used in grinding and polishing as rapidly or as much as does balsam.

The procedure used by Chase is indicated below:

- (1) Fix the part from which the section is to be taken as desired.
- (2) Wash according to the method of fixation used, and bring to a syrupy aqueous solution of gum arabic (or dextrin).
- (3) Freeze on the freezing chamber of a freezing microtome.
- (4) Cut a thin slice or slices, using a very fine saw. (Williams<sup>10</sup> advises the use of a fine jeweler's saw.)
- (5) Wash the slice in water to remove the gum arabic.
- (6) Stains which can be applied in toto may be used here, e.g., carmine, hematoxylin, etc.
- (7) Ascending alcohol series to 95 per cent: thirty minutes to several hours each, according to the size of the slice.
- (8) Acetone: thirty minutes or more.
- (9) Place in a thin solution of celluloid (about the consistency of table syrup), using a depth of solution at least twice the thickness of the slice. A very

<sup>8</sup> Hopewell-Smith, A. *Dental Microscopy*. Ed. 2, Phila., 1914, p. 48.

<sup>9</sup> Chase, S. W. J. *Dental Research*, 10: No. 3, 1930.

<sup>10</sup> Williams, A. *Dental Cosmos*, 69: 715-721, 1927.



convenient form of dissolved celluloid is the clear celluloid lacquer obtainable at any paint store, e.g., Duco, clear; Rogers Brushing Lacquer, clear, etc.

(10) Leave the container open a mere crack, and allow the solvent of the lacquer to evaporate slowly at room temperature, until it will scarcely flow when the container is tilted steeply; then remove the slice with a considerable amount of surrounding celluloid solution to a smooth flat sheet of heavy lead foil (Boedeker, 1926, forms the container of lead foil), and allow to evaporate further until the celluloid matrix is completely hardened.

(11) Grind and polish one face of the slice (in the presence of water) to the desired plane of section. Wash and wipe clean.

(12) Attach to grinding mount with Household Cement as already described.

(13) Grind and polish the section to the desired thickness (in the presence of water). Wash and wipe clean.

(14) Soak in acetone to remove the celluloid, and transfer to slide.

(15) Flood with xylol to remove acetone.

(16) Mount in Canada balsam.

Note: Various staining methods may be applied by the drop method between steps 14 and 16.

**4. Decalcification.** Decalcification with hydrochloric or nitric acids is widely used to prepare specimens consisting of hard and soft tissues so as to retain the natural relations of their parts. The chief, or only, disadvantage is the fact that this entails the loss of enamel. For most purposes however this is unimportant. Before decalcifying, the pieces should be cut with a fine-toothed jeweler's saw into as thin slices as possible without distorting or tearing the parts to be studied. If it be desired to study the pulp in situ, it is best to grind away the enamel and dentine on a stone, using plenty of water, until the pulp is almost exposed in one or two places. The specimen is then fixed before putting it into the decalcifying solution. A relatively large volume of this should be used, and the specimen should be exposed to its action no longer than necessary.

Seidel has devised a special jar for decalcification, which seems to possess some advantages. It consists of a glass cylinder which has an outlet tube at the bottom and an inlet tube at top. Its cover is ground to form a tight joint. At half its height the cylinder is constricted to support a perforated glass plate on which the specimen is placed. The decalcifying fluid can be withdrawn and replenished with a minimum of disturbance to the specimen. Likewise the washing and dehydrating following decalcification can be performed.

After decalcification the specimen may be imbedded either in paraffin, celloidin or celloidin and paraffin. In general the latter method of imbedding is much to be preferred.

**5. Paraffin Technique.** a. Manley<sup>11</sup> suggested the following method:

<sup>11</sup> Manley, E. B. *Brit. Dental J.*, 60: 456, 1936.

- (1) Fix in 10 per cent saline-formol, forty-eight hours.
- (2) Decalcify in 5 per cent nitric acid, use a large bulk of fluid and change frequently or use an agitator. (It is important to obtain complete decalcification; test with a new sharp needle and remove the tooth from the acid as soon as decalcification is complete. Four to eight days are usually sufficient.)
- (3) Wash in running water twenty-four to forty-eight hours.
- (4) Dehydrate in ascending grades of alcohol commencing with 50 per cent, twelve hours.
- (5) 70 per cent alcohol, twelve hours.
- (6) 90 per cent alcohol, twelve hours.
- (7) Absolute alcohol (1) twelve hours.
- (8) Absolute alcohol (2) twelve hours.
- (9) Absolute alcohol (3) twelve hours. (Thorough dehydration is very important and care should be taken to drain the vessel at each change of solution.)
- (10) Clear in xylol, removing as soon as clear; usually not more than twelve hours is required.
- (11) Transfer to paraffin wax 54°C. and leave for twelve hours.
- (12) Fresh paraffin, twelve hours.
- (13) Imbed in new paraffin, with the tooth diagonally across the block.
- (14) Sections may be cut on the Cambridge rocking microtome, using a very sharp flat razor.
- (15) Sections are mounted on slides by the albumin-glycerin and water method and dried in the oven at 37° to 40°C.

b. The method which follows has been successfully used in our laboratory for the study of dentine and pulp in situ.

(1) Place a freshly extracted tooth in 4 per cent formalin for twelve to fourteen hours. Clip the ends of the roots or drill a hole through the hard structure so that the formalin will reach the pulp.

(2) Remove, dry on towel and touch the clipped ends or other openings to the pulp, with liquid celloidin in order to seal.

(3) Dry quickly and place in a half-gallon bottle of decalcifying solution (10 per cent hydrochloric acid C.P.) for ten days to two weeks or longer, trying with a sharp needle from the fifth day on until entire specimen can be easily pierced.

(4) Remove and wash in running water for twenty-four hours.

(5) Place in 95 per cent alcohol for twenty-four hours.

(6) Absolute alcohol five hours.

(7) Chloroform one hour.

(8) Chloroform and paraffin (melting point 45°C.) equal parts, on top of oven in ground-glass stoppered bottle over night (oven 58°C.).

(9) Place in paraffin bath inside of oven. (Three baths one-half hour each for teeth, other tissues one hour. The melting points of the baths are 1: 42-46°, 2: 52-56°, 3: 58-60°.)

(10) Prepare the imbedding material by mixing 235 c.c. of paraffin (52-56°) with 15 c.c. of beeswax. Pour imbedding material in suitable boats and imbed

specimen. When film of wax appears on top then sink boats in ice water to allow air bubbles to escape from tissues. Do not disturb for twenty to thirty minutes.

(11) Chill knife and specimen with ice water before cutting.

(12) Cut and float sections on top of luke warm water (37°).

(13) Place sections on slide previously smeared with Mayer's egg albumen.

(14) Arrange sections in Pillsbury boxes and allow them to remain in oven over night at 58°.

They are now ready for staining.

**6. Celloidin Technique.** The following method is a modification of the technique developed by Mr. Basil B. Varian, technician in the Department of Anatomy, University of Pennsylvania, and has been carried out successfully in our work.

*Fixing and Hardening:* Fix in 4 per cent formalin. Add a buffer to the stock solution in order to counteract the acid present in the commercial product.

The fixing fluid must have full access to the soft tissues. This end is accomplished by cutting off the apex of the tooth or drilling a hole through the crown to the pulp chamber. The time of fixation varies with the size of the specimen. In cases of single teeth, fix forty-eight hours. When fixing larger specimens containing teeth, bone and soft structures, the fixing time should be prolonged accordingly. Place in 80 per cent alcohol.

#### *Imbedding:*

*Preparation of Parlodion:* Rinse shreds of parlodion three times in distilled water. Place in oven over night. A 12 per cent solution is made as follows:

Equal parts of absolute alcohol and ether are used as a solvent for the parlodion. (Absolute alcohol and ether are kept in separate containers, the bottom of which should contain anhydrous copper sulphate.) Various percentages of parlodion in solution are made up from this 12 per cent solution.

Solution	Parlodion
No. 1 . . .	1½ per cent
No. 2 . . .	2 per cent
No. 3 . . .	5 per cent
No. 4 . . .	7 per cent
No. 5 . . .	10 per cent
No. 6 . . .	12 per cent

*Procedure for imbedding a specimen consisting of teeth, bone and soft tissues:*

(1) From the 80 per cent alcohol the tissues are placed in .95 per cent alcohol two weeks or longer, depending on the size of the specimen.

(2) Absolute alcohol two weeks or longer.

(3) Absolute alcohol which has been exposed to anhydrous copper sulphate to insure absolute dehydration, two weeks or longer.

(4) Absolute alcohol and ether equal parts (both are exposed to anhydrous copper sulphate before mixing) two weeks or longer.

(5)  $\frac{1}{2}$  per cent parlodion one month or longer.

(6) 1 per cent parlodion one month or longer.

(7) 2 per cent parlodion one month or longer.

(8) 5 per cent parlodion one month or longer.

(9) 7 per cent parlodion one month or longer.

(10) 10 per cent parlodion one month or longer.

(11) 12 per cent parlodion one month or longer.

(12) Thick parlodion is poured into a stender dish and tissue transferred from the 12 per cent parlodion to this thick solution. The height of the parlodion in the vessel should be twice that of the tissue to insure perfect imbedding. The tissue is oriented in the position desired for cutting. The lid of the stender dish is placed on tightly to allow bubbles from the tissue to rise to the surface and thus permit even evaporation for twenty-four hours or longer. If bubbles are still present move tissue gently with the aid of needles to allow them to escape.

(13) Place a thin strip of paper between lid and dish twenty-four hours or longer.

(14) Evaporation takes from five to seven days or more. The slower the evaporation the better the results. Evaporation is continued until the parlodion has the consistency of hard rubber.

(15) Place the block in 80 per cent alcohol for forty-eight hours or longer until ready for decalcification.

(16) Trim block to suitable size making certain that the whole tissue is surrounded by sufficient parlodion to insure proper cutting.

#### *Decalcification:*

(1) Place block which has been in 80 per cent alcohol, in 10 per cent acetic or hydrochloric acid dissolved in 70 per cent alcohol (10 parts concentrated  $C_2H_4O_2$  or HCl in 90 parts 70 per cent alcohol).

(2) Change daily for three weeks or longer until the specimen can be easily pierced with a needle.

(3) After sufficient decalcification has taken place, spaces will be noticed in the parlodion where the calcified parts were present. Canals are drilled in the parlodion to reach these spaces.

(4) Block washed twenty-four hours or longer in running water to remove decalcified matter.

(5) Block now washed for twenty-four hours in a weak solution of sodium bicarbonate or lithium carbonate to neutralize the acid.

(6) Washed in water twenty-four hours or longer.

(7) Block placed in 50 per cent alcohol twenty-four hours or longer.

(8) Block placed in 70 per cent alcohol twenty-four hours or longer.

(9) Block placed in 80 per cent alcohol twenty-four hours or longer.

- (10) Block placed in 95 per cent alcohol one-half hour or longer.
- (11) Block placed in absolute alcohol one-half hour.
- (12) Block placed in alcohol-ether five to twenty minutes.
- (13) Block placed in  $\frac{1}{2}$  per cent parlodion five to twenty minutes.
- (14) Block placed in 12 per cent parlodion five to twenty minutes.
- (15) Block placed in chloroform vapor twenty-four hours or longer to harden.
- (16) Block placed in 80 per cent alcohol twenty-four hours or longer until ready for mounting.

#### *Mounting on Fiber Block:*

- (1) Block removed from 80 per cent alcohol is now placed in 95 per cent alcohol for five minutes.
- (2) Absolute alcohol for five minutes.
- (3) Ether-alcohol for five minutes.
- (4) Thick parlodion for five minutes.
- (5) Block now transferred to the serrated surface of a fiber block previously soaked in ether-alcohol. Make certain that no air bubbles are present between parlodion block and fiber block.
- (6) The mounted block immediately transferred to chloroform vapor for twenty-four hours to harden.
- (7) Placed in 80 per cent alcohol for one-half hour before cutting or indefinitely until ready for cutting.

#### *Cutting:*

- (1) Block is transferred to cutting machine and fastened tightly, making certain that parlodion is always kept moist with 65 per cent alcohol.
- (2) Knife is set as obliquely as possible without endangering the cutting of the whole section.
- (3) Knife and block are kept continuously soaked in 65 per cent alcohol. As each section is cut it will be noticed that it curls. It is flattened out over the knife with the aid of a brush. The section is cut almost to the end of the plane allowing a small piece of the same to remain uncut until the curled portion is brushed flat. When this is done the remaining attached part is cut through. Cutting is done slowly.
- (4) If a serial arrangement is required the sections are placed in 80 per cent alcohol and mounted in the order desired. If no serial sections are necessary the sections are carried to a container with 80 per cent alcohol and allowed to remain there until ready for mounting on slides.

#### *Slide Mounting:*

- (1) Clean glass slide must be used. A few drops of the following mixture is smeared evenly over the slide:

Strained egg albumen.....	50 parts
Glycerin C.P. ....	50 parts
Crystal of camphor	

(2) With the aid of a spatula, held at the edge of the knife, the sections are transferred to a slide in the proper serial order.

(3) Each time a section is transferred to a slide the spatula must be touched to a towel to remove excess alcohol.

(4) When a sufficient number of sections are transferred to a slide they are pressed firmly to it by means of fine filter paper.

(5) Flood slide immediately with ether-alcohol solution, allowing it to act for several minutes until some evaporation has taken place and the parlodion is becoming dry. Do not, however, allow the specimen to dry completely.

(6) Press firmly with filter paper.

(7) Immediately upon removal place in 95 per cent alcohol for one hour. If sections are not to be stained immediately they are placed in 80 per cent alcohol until time for staining. However, the slide remains in the 95 per cent alcohol for at least one hour.

A section prepared in this fashion will take any stain except aniline or vital stains.

#### *Oil of Cloves Method of Mounting Sections<sup>12</sup>:*

Same procedure as above under "Slide Mounting" (1) to (4) inclusive.

(5) Immediately flood slide with oil of cloves. (Use twice-rectified oil of cloves.) Keep sections in same for several hours or days until they are cleared.

(6) Place in 95 per cent alcohol several hours to remove the oil of cloves. Proceed as under (7).

#### *Preparation of Mounted Slides Prior to Staining:*

When oil of cloves method for mounting sections is preferred proceed as follows:

(1) 95 per cent alcohol, five to ten minutes.

(2) Ether-alcohol solution, five to ten minutes (to make certain that all the parlodion is removed).

(3) 95 per cent alcohol, five minutes.

(4) 80 per cent alcohol, five minutes.

(5) 60 per cent alcohol, five minutes.

(6) Thiosulphate solution, five minutes.<sup>13</sup>

(7) Distilled water.

(8) Stain (any stain can be used when the oil of cloves method for mounting sections is used).

When oil of cloves method is not used for mounting sections the following procedure is followed:

<sup>12</sup> This method is longer but removes the parlodion.

<sup>13</sup> Sodium thiosulphate solution is made as follows: Stock solution of 2.5 per cent sodium thiosulphate in distilled water. When sections are to be subjected to sodium thiosulphate we dilute the 2.5 per cent stock solution 10 times.

- (1) From the 80 per cent alcohol the section is placed in 95 per cent alcohol for five to ten minutes.
- (2) 80 per cent alcohol, two minutes.
- (3) 70 per cent alcohol, two minutes.
- (4) 60 per cent alcohol, two minutes.
- (5) Sodium thiosulphate solution until section becomes completely white (approximately five minutes).
- (6) Then place slide in distilled water. Sections are now ready to stain with any stain except vital and aniline stains.

**7. Celloidin-Paraffin Technique of Burket.<sup>14</sup>** A technique has recently been perfected by Burket, a member of the Dental Study Group at Yale University. This technique enables one to cut sections of the dental tissues and at the same time to preserve the relations of the tooth to the jaw.

"Available methods for sectioning single teeth were not entirely adapted to cutting human teeth in situ. In most instances large quantities of the material on the external surface of the enamel and in the gingival crevice were lost in the decalcifying and imbedding procedures and the thickness of the sections limited the study to the more gross changes.

"Experimental studies were carried on by Arnim<sup>15</sup> for two years and were directed primarily to the preparation of histological material derived from studies on the rat. Many facts arising out of this work have been made available for the studies on the tooth of man. The methods for sectioning animal material have been published elsewhere.

"The basis for the procedures which follow was the combination of the methods of Guild<sup>16</sup> and Williams.<sup>17</sup>

**"Fixation:** For convenience and simplicity the tissues were fixed in 10 per cent formalin, changed weekly for two or three weeks. Fixatives containing acetic acid could not be used, as it was desired to retain in situ the enamel cuticle and as much of the enamel as possible.

**"Division of the Tissues:** The formalin-fixed material was thoroughly washed in tap water for twenty-four hours. The specimens were then cut into "blocks" by means of a thin rotary saw containing one or more teeth. Each block was wrapped and tied in gauze with its identification number enclosed.

**"Dehydration:** The specimens were dehydrated in each of the following solutions for twenty-four hours: 60 per cent, 80 per cent, two changes of 95 per cent, two changes of absolute ethyl alcohol, and two changes of equal parts absolute alcohol and ether. All stock absolute alcohols, as well as the low percentage paralodions, were kept over anhydrous copper sulphate. Dehydration with all abso-

<sup>14</sup> Burket, L. W. Dissertation, Yale Univ., 1936.

<sup>15</sup> Arnim, S. S. *Anal. Record*, 62: 321, 1935.

<sup>16</sup> Guild, S. R. *J. Lab. & Clin. Med.*, 4: 153, 1919.

<sup>17</sup> Williams, A. *Dental Cosmos*, 67: 715, 1927.

lute alcohols and ether-alcohols was carried out in 6 inch desiccating jars in which anhydrous copper sulphate was placed in the desiccating chamber and separated from the specimens by a double layer of filter paper and a perforated porcelain plate. A small air-driven windmill, running through the cork in the lid of the desiccator, assured constant movement of the solution. When the copper sulphate in the base of the desiccator turned light blue, it was replaced.

*"Infiltration:* Each specimen and record number were placed in a screw-top bottle after its gauze wrappings had been removed. The tissues remained in 1 per cent parlodion for six weeks, 3 per cent parlodion for four weeks and 6 per cent parlodion for four weeks.

*"Imbedding:* The specimens were imbedded in large stender dishes. The identification paper was placed on the bottom of the imbedding dish, written side down. A small quantity of 8 per cent parlodion was poured in and the mesial surface of the specimen placed over the identification paper. The imbedding dish was then filled with 8 per cent parlodion, which was allowed to harden for several days. This could be hastened by placing the imbedding dishes in a closed vessel containing a small amount of chloroform. When hard, the parlodion was trimmed to leave a minimum of material on all surfaces of the specimen. Bubble formation in the parlodion was avoided if the room temperature was not too high or the specimens were not allowed to remain in direct sunlight.

*"Hydration:* The trimmed specimens were placed in as many changes of chloroform as was necessary until they sank to the bottom of the vessel. They were then immersed for twenty-four hours respectively in absolute iso-propyl or butyl alcohol, 95 per cent iso-propyl or butyl alcohol, 80 per cent and 60 per cent ethyl alcohol. The absolute alcohols were used in desiccators as previously described. Iso-propyl or butyl alcohols were used after the original imbedding as parlodion is insoluble in them. The absolute iso-propyl was prepared from the 98-99 per cent grade by the use of copper sulphate.

*"Decalcification:* After the specimens were washed for twenty-four hours in running tap water, they were placed in a decalcifier. Five per cent nitric acid, siphoned from a 3 gallon storage jar, was run through glass tubing fitted with a drip cock to the bottom of the decalcifier. The rate of flow was determined by the number of specimens being decalcified. Approximately 1 gallon of acid mixture was used every twenty-four hours when decalcifying specimens equivalent to a complete maxilla and mandible. The specimens were placed on a perforated porcelain shelf about 2 inches above the bottom of the decalcifier. An over flow tube near the top of the jar was used to conduct the acid waste into a crock. The test used to determine if the specimen was decalcified depended on the presence of the Ca-ion. The specimen to have been tested was placed for twelve hours in 50 c.c. of unused 5 per cent nitric acid. At the conclusion of the twelve hours, 5 c.c. of this solution was placed in a test tube with 2 drops of methyl red, to which ammonium hydroxide was added drop by drop until the indicator changed to a permanent yellow color. Two cubic centimeters of saturated ammonium oxalate were added and the mixture allowed to stand



for one hour. If a precipitate of calcium oxalate was formed, the specimen was not sufficiently decalcified and was replaced in the decalcifier and tested again in several days. The decalcified specimens were placed in two changes of 5 per cent sodium sulphate for twenty-four hours each. This tended to neutralize the acid, prevent the swelling of the tissue and serve as a mordant. (Shafer.<sup>18</sup>)

*"Second Dehydration:* After the specimens were thoroughly washed in running tap water for at least twenty-four hours, they were allowed to remain in each of the following solutions for twenty-four hours: 60 per cent, 80 per cent ethyl alcohol, 95 per cent butyl alcohol and two changes of absolute butyl alcohol.

*"Reimbedding:* The specimens were placed in an imbedding dish with the identification paper side on the bottom of the dish, 6 per cent parlodion was poured on and allowed to remain until the space formerly occupied by the enamel was filled with parlodion. The blocks were hardened over chloroform as a final procedure. After the specimens had been trimmed with at least 4 mm. of parlodion remaining on all sides they were placed in chloroform until they sank to the bottom of the vessel. Often several changes of chloroform were required.

*"Paraffin Infiltration:* The imbedded specimens were then cleared in benzene. Large quantities and frequent changes of this clearing medium were essential. Usually four changes of twenty-four hours each were required before the block became transparent. The specimens were then placed in two changes of low melting paraffin (Grübler 40-42°C.) of twenty-four hours each. The use of this low melting paraffin allowed the specimens to be left in the liquid medium for a period long enough to insure thorough infiltration and yet not cause an excessive hardening of the dentine. They were then placed in a similar paraffin containing 1 to 10 per cent bayberry wax. The addition of the bayberry wax increased the hardness of the low melting paraffin so that it was comparable to the higher melting point (56°C.) paraffins. The specimens were removed from the bayberry paraffin, allowed to cool and were finally stored in the ice box. The identification paper was still visible and the use of tags was entirely eliminated in the technique.

*"Cutting:* The blocks were fastened to wood or fiber mounts with high melting point paraffin and bayberry wax, the surface containing the identification paper being kept uppermost. Hence the initial cutting surface was the mesial surface of the specimen. Serial sections were cut on a Leitz No. 1200 microtome at 12 micra using the wet method with 80 per cent alcohol solution on the knife. The longest possible cutting edge of the knife was used. The angle of the knife relative to the cutting plane was between the third and fourth mark on the knife holder. The sections were unrolled on the knife and carefully smoothed out with a soft camel's hair brush moistened with the 80 per cent alcohol solution mentioned above. Care was taken not to touch the tissue with the brush. If the block was not infiltrated perfectly the sections were carefully drawn up flat on the knife with a soft camel's hair brush, as they were being cut. The tissues were removed on small consecutively numbered squares of tissue paper

<sup>18</sup> Schafer, E. S. The Essentials of Histology. Phila., Lea and Febiger, 1929, p. 589.

and placed section side down in small dishes filled with 80 per cent alcohol.<sup>19</sup> A narrow strip of white paper was inserted after every tenth section to serve as a marker in the mounting process.

*"Affixing:* The paraffin-parlodion imbedding method complicated the mounting of every tenth section prior to staining. Provided the following steps were carefully followed, the sections could be mounted successfully without resorting to any celloidin coating methods, which at their best interfere with the study under high magnification and cause wrinkling and displacement of the thin sections. The slides (50 mm. x 75 mm.), thoroughly cleaned, were stored in 95 per cent alcohol and dried as needed with a paper towel. After they were marked with a diamond glass pencil, they were wiped again. The slides as used were lightly coated with egg albumen. A fresh mixture of 4 parts fresh egg albumen with 1 part glycerin without any preservative was found most satisfactory. This was kept in the refrigerator when not in use and discarded after four days.<sup>20</sup> Every tenth section as indicated by the narrow white paper markers, was successively placed in a large Petri dish containing 60 per cent alcohol and floated off the tissue paper. It was then passed through 30 per cent alcohol and into a dilute acetic acid solution containing 3 drops of glacial acetic acid to 300 c.c. of distilled water. The sections were lifted from the acidulated water by means of a silver section carrier and the excess water removed by touching the edge of the carrier to a paper towel before transferring the tissue to the slide. The sections were arranged on the slide and the excess water drained off. They were covered with a small rectangle of hard-surfaced filter paper moistened with 80 per cent ethyl alcohol. This was pressed down firmly over the sections and was blotted with another piece of filter paper. The papers were then removed carefully by rolling them back over one finger at a time. A dry paper was placed over the sections and moistened with absolute iso-propyl alcohol from a dropping bottle. The slide was blotted again and the papers were carefully removed as before and another dry one placed over the sections, which was in turn flooded with benzene. This partially deparaffinized the sections. A new piece of filter paper was moistened with 80 per cent alcohol. The benzene moistened paper was blotted and quickly removed, and the one previously moistened with 80 per cent alcohol was rapidly applied. It was firmly and carefully pressed down over the sections. The paper was removed and the slide placed in 80 per cent alcohol until time of staining. Lateral movement of the applied papers was avoided. The different reagents were allowed to remain approximately one minute on the sections.

*"Staining:* The sections were deparaffinized by carefully passing the slide basket containing the slides through 95 per cent iso-propyl alcohol, absolute iso-propyl alcohol and two changes of benzene; hydrated by passing them through a second absolute iso-propyl alcohol, a second 95 per cent iso-propyl

<sup>19</sup> A numbering machine and Bates ink were used. This ink is not soluble in the reagents used.

<sup>20</sup> Suggestion of Mr. Meyrowitz, Technician, Dental Study Group, at The School of Medicine of Yale University.

alcohol, 80 per cent, 60 per cent, 30 per cent, 15 per cent ethyl alcohol, and two changes of distilled water. The slides were allowed to remain approximately three minutes in each solution. If the sections were found to stain poorly they were "freshened" at this point. The slides were placed in 20 to 30 per cent hydrogen peroxide solution for fifteen minutes to a half hour. After two changes of distilled water, the sections were ready to be stained with Delafield's hematoxylin, 20-25 drops in 300 c.c. of distilled water for a full slide basket, overnight. The weak staining solution offered greater opportunity for the absorption of the stain by the tissues having an affinity for it. The staining qualities of old Delafield's hematoxylin can be satisfactorily restored by the addition of a saturated aqueous solution of ammonia alum. (Mallory and Wright, Ed. 6.) The slides were carefully passed through two changes of distilled water and were differentiated in an acid-aqueous solution containing 4 drops of concentrated hydrochloric acid to every 100 c.c. of distilled water. The slides were then passed through distilled water to two changes of basic tap water where they were allowed to remain until a blue color developed. A lithium carbonate or dilute ammonia solution could be substituted for the tap water, if the results were not satisfactory by the former method. Following this the slides were passed through distilled water or washed with slightly basic tap water. They were counterstained with a dilute (1 per cent) alcoholic eosin solution, passed through two changes of 95 per cent iso-propyl, 98-99 per cent iso-propyl alcohol and then into beechwood creosote. To reinforce the eosin the sections were often placed in beechwood creosote containing a few grains of eosin. They were then removed from the clearing solution, laid flat, flooded with xylol, and then blotted with small rectangles of filter paper and coverslips were applied by the use of medium thick balsam (neutral in xylol). The slides were then placed in a 37°C. incubator for a week to ten days to harden the balsam. They were then cleaned with a mixture of xylol and 95 per cent alcohol. The slides were numbered with India ink over which sandarac varnish was applied to prevent smudging.

"Several sections of satisfactory thinness, in which the gingival debris, enamel cuticle and debris could be retained with moderate success, were obtained by the above technique. It became evident that, for a more complete picture of the pathological changes, especially those associated with the beginning of carious lesion, the so-called 'organic' matrix of the enamel must be retained in situ on the dentine. Previous workers (Malleison,<sup>21</sup> Chase<sup>22</sup> and Boedeker<sup>23</sup>) had developed methods of retaining this material in isolated fragments of the enamel 'entirely free from the dentine.' No reference could be found concerning its retention on the dentine in the adult erupted tooth. The methods used by these workers consisted essentially in decalcifying the small enamel fragments in an acidulated imbedding media such as celloidin. In such a solution ionization of the acid was slight and decalcification slow. This method, if applied to individual teeth or teeth in situ, afforded no means of retaining the normal relationship between the enamel and dentine.

<sup>21</sup> Malleison, H. C. *Brit. Dental J.*, 66: 907, 1925.

<sup>22</sup> Boedeker, C. F. *Ztschr. f. wiss. Mikr.*, 22: 190, 1905.

<sup>23</sup> Chase, S. W. *Anat. Rec.*, 38: 239, 1927.

*"Modification of Technique for Retention of Enamel Matrix:* The double parlodion-paraffin imbedding technique routinely used preserved the enamel cuticle and lamellae in good anatomical relationship. The problem was limited, therefore, to the retention of the enamel remains in position. This was approached by placing specimens carried to the decalcifying stage in various concentrations of different acids. Large quantities of enamel remains were retained on the teeth decalcified in 5 per cent acetic acid. Further experimentation revealed that 7 per cent acetic acid was more satisfactory than other concentrations of acetic acid or trichloroacetic acid. An attempt was made to modify the quantitative test for calcium reported by Arnim for use with human teeth in situ, but the large difference in the quantity of calcium in the different human teeth made this extremely difficult. Decalcification was usually determined by testing the specimen with a needle. Aside from the changes in the decalcifying technique and the use of extreme care in the reimbedding, the regular technique was followed."

**8. Dextrin Imbedding and Frozen Sections.** Instead of imbedding in celloidin the tissues may be imbedded in a dextrin solution and cut on the freezing microtome. This method has been developed by Hopewell-Smith.<sup>24</sup> In experienced hands it yields beautiful isolated sections and in time it is somewhat shorter than celloidin imbedding. However, serial sections and exact control of thickness are impossible. After fixation the soft parts and apices of the roots are dried on a cloth, and a large drop of thin celloidin placed on them, so that in a few moments a thick film covers them over, and protects them from the action of acid reagents, e. g., as in decalcification. Afterward the celloidin film is removed by immersion in a watch-glass of ether for about five minutes, followed by careful picking or rubbing with a brush, and the specimens are neutralized with sodium or lithium bicarbonate (5 grains to an ounce of water). They are then trimmed to appropriate size and shape, washed well in water, and immersed in a saturated solution of dextrin for at least fifteen hours. The pieces of tissue are finally placed on the stage of an ether-freezing microtome and cut in the ordinary manner.

## B. EXAMINATION OF SPECIAL STRUCTURES IN DENTAL HISTOLOGY

**1. The enamel cuticle (Nasmyth's membrane).** Suitable specimen teeth are procured and treated in the following manner:

- (1) Wash and brush the specimen in tap water.
- (2) Fix in 4 per cent neutral formalin for twenty-four hours.
- (3) Wash in tap water for twelve hours.
- (4) Stain in Mallory's aniline blue for twenty-four hours.

<sup>24</sup> Hopewell-Smith, A. *Dental Microscopy*. Ed. 2, Phila., 1914, p. 5.

- (5) Wash and brush specimen in tap water.
- (6) Place specimen in 10 per cent hydrochloric acid for ten minutes. As the enamel goes into solution, there will be seen surrounding the crown of the tooth a delicate, whitish, opaque, partly attached membrane (the enamel cuticle).
- (7) Tease off membrane with needles and brush.
- (8) Gather up fragments of enamel cuticle on slide coated with egg albumen.
- (9) Blot fragments with filter paper.
- (10) Wash in 5 per cent solution of sodium thiosulphate or bicarbonate of soda for ten minutes.
- (11) Wash in tap water for ten minutes.
- (12) Run through alcohols.
- (13) Xylol.
- (14) Mount in Farrant's medium (p. 617) or gum damar.

Nasmyth's membrane, when present, is also demonstrable by Boedeker's method (see blow).

**2. Isolated Enamel Rods.** These can be simply demonstrated by immersing the tooth in 5 to 10 per cent hydrochloric acid solution. Examine after twenty-four hours. If the enamel is quite soft, remove a small portion with a needle to a slip, and tease it out. Mount in a drop of physiological salt solution under a cover glass. A carmine stain can be run in from the side and later be washed out by applying a blotter to one edge of the cover glass and running acidulated water (10 per cent acetic) in from the other side. A permanent preparation may be then made by "ringing."

The structure and relations of the individual enamel prism have long been studied by direct microscopic observation of ground sections during the progress of decalcification.<sup>25</sup>

Dilute acids are run "under a coverslip supported on fine cover-props over a thin ground section of enamel. The acid can be continuously changed by employing a piece of blotting paper at the opposite side of the coverslip from that at which the acid is supplied. One per cent nitric, hydrochloric, or sulphuric acids or 5 per cent chromic, acetic, citric, or lactic acids decalcify slowly enough so that all the steps in the process of decalcification can be observed without gas being formed sufficiently rapidly to obscure the picture. The process may be stopped at any stage desired by supplying water instead of acid, and, if sufficient care be used, the section can be stained and mounted in balsam without disturbing the position of the remains."

### 3. Structure and Chemical Composition of Enamel.

*Boedeker's Method.* Boedeker<sup>26</sup> has devised a "celloidin-decalcify-

<sup>25</sup> Chase, S. W. *Anat. Rec.*, 36: 239, 1927.

<sup>26</sup> Fundamentals of Dental Histology and Embryology. N. Y., 1926, p. 223.

ing method" to demonstrate minute quantities of organic matter in the enamel.

A small piece of enamel (0.5 to 1 mm. thick), entirely freed from dentine, is passed through the alcohols for dehydration, ten minutes each; then into methyl alcohol for one to two hours and finally into acid celloidin (parlodion) (20 c.c.). This is prepared as follows: Thoroughly dried chips of parlodion (Dupont) are dissolved in c.p. methyl alcohol to make a thick syrupy solution. To 150 c.c. of this add the following mixture drop by drop, constantly stirring with a glass rod:

Nitric acid, C.P. ....	10 c.c.
Methyl alcohol .....	40 c.c.

The glass dish in which the enamel is decalcified should have an airtight cover. This dish should be moved as little as possible and then only with the greatest care. The progress of decalcification should be checked occasionally microscopically without removing the enamel from the acid-celloidin. The organic matrix of the enamel will become discernible after ten to twelve hours as a brown spongy substance.

When the entire specimen has acquired this appearance, decalcification is complete, usually within two to seven days. Then uncover the dish to allow the celloidin to harden. Cut out specimen, surrounded by a narrow margin of celloidin and pass this block into 70 per cent alcohol, one to two hours; 40 per cent alcohol, one to two hours; watery solution of alum, twenty-four hours; running water, six to twelve hours; ascending alcohols, one to two hours in each; do not use absolute; aniline oil, six to twelve hours (when cleared block becomes brown and perfectly transparent); aniline, chloroform, equal parts, three to six hours; chloroform, six to twelve hours; imbed in paraffin (m.p. not above 52°C.) two hours, cut sections on microtome, 3 $\mu$  to 10 $\mu$ ; spread sections on slips; dry; decerate in xylol, three minutes; dissolve parlodion in ether-alcohol (ethyl), equal parts; pass through absolute alcohol, one minute; through descending alcohols to water; stain (Boedeker prefers Heidenhain's ferric hematoxylin); dehydrate; mount in balsam.

Chase<sup>27</sup> has used Boedeker's or Malleeson's method to study enamel prisms and interprismatic substance. He, however, shortened the period of decalcification to about one-quarter (or less) of that described.

**4. The Structure of the Enamel.** The manner in which the structural peculiarities of the enamel are related to its organic content, permeability and the possibility of post-eruptive changes is one of the most vital questions in dental histology. A number of methods have been devised to attack this problem.

*a. Immersion of Apex of Tooth in Stain.* V. Beust<sup>28</sup> cemented freshly

<sup>27</sup> Chase, S. W. *Anat. Rec.*, 36: 239, 1927.

<sup>28</sup> v. Beust, T. *Dental Cosmos*, 54: 659, 1912.

extracted teeth in the corks of bottles containing a strong alcoholic solution of fuchsin with NaCl to accelerate capillary flow, in such a manner that only the tip of the root reached the stain. In time the enamel, and in many cases the enamel cuticle, became stained.

*b. Injection of Stain into Pulp Canal under Pressure.* He also performed the following experiment:

The nozzle of a syringe is securely cemented into the enlarged apical opening of a root with a very large pulp cavity, from which the pulp has been removed, and is completely filled with staining solution. A wire is repeatedly inserted and withdrawn in order to expel all the air. With the fingers, or with rubber bands, pressure is exerted upon the piston. In a short time, varying from a few minutes to a few days according to the pressure and the density of the tooth, the staining solution will be perceptible in all parts of the external tissue. Ground sections are made of such teeth.

*c. Silver Nitrate ( $\text{AgNO}_3$ ).* Later, v. Beust<sup>29</sup> used silver nitrate as the "staining" solution. He proceeded as follows:

The root of a freshly extracted tooth is sawed through about midway between the apex and the crown, and the root and pulp cavity enlarged to receive the end of a glass funnel or pipette. The surface of the root is superficially ground, up to the enamel line, and the sawed end and ground surface shellacked, care being taken to prevent the varnish from reaching the inner part of the pulp cavity or the surface of the crown. The funnel, containing a wire, is now sealed into the tooth with sticky-wax, allowing the wax to spread over the shellacked part of the tooth. After the wax has cooled, a few drops of water are placed in the funnel and the wire withdrawn (to exclude the air). One per cent aqueous silver nitrate solution is now added and the specimen is placed in the dark. After the lapse of a few days the tooth is ground in paraffin oil, the section is wiped and placed in a bottle containing dilute alcohol. The bottle is now placed in the sun until decomposition of the salt has taken place, whereupon the specimen is passed through strong and finally through absolute alcohol, washed in xylol and imbedded in balsam.

*d. Williams' Silver Nitrate ( $\text{AgNO}_3$ ).* Williams<sup>30</sup> has studied the enamel very carefully for many years.

An entire tooth is immersed in a 2 to 5 per cent solution of silver nitrate and left in it from one week to six months. Long-continued staining will often reveal facts not shown by brief staining. The tooth is then cut, ground and polished as previously described (p. 353) to the thickness of a single enamel rod.

During the grinding the specimen must be examined from time to time in the following way: Clean it and brush it over for thirty seconds or so with a 3 per cent solution of lactic acid. This will remove a little of the cement substance

<sup>29</sup> v. Beust, T. *Dental Cosmos*, 56: 201, 1914.

<sup>30</sup> Williams, J. L. *Dental Digest*, 31: 827, 1925.

between the rods and also around the organic substructure in the rod, thus revealing something of the real nature of the tissue. Wash off the lactic acid, cover with a drop of distilled water and cover glass, and examine.

All study and all photography should be done with distilled water for a mounting medium. Permanent preparations may be made by mounting in balsam.

*e. Staining Enamel in Bulk.* Attempts have been made to stain enamel in bulk.

For example Leigh<sup>31</sup> has obtained the best results from the use of freshly extracted teeth, eliminating formalin from the technique entirely, passing them through graduated solutions of alcohols, and then immersing them in one of the following stains: (1) 2 per cent alcoholic solution of basic fuchsin or gentian violet or equal parts of crystal violet and brilliant green; (2) 10 per cent aqueous solution of c.p. silver nitrate. The roots of the teeth were cut off to permit free entrance of solutions into the pulp chamber, and the solutions diffused through the dental tissues. Re-section of the root also obviates a high liability of the microscopic sections to longitudinal fracture. A varnish of celloidin was used on the exterior of the enamel in certain cases to prevent the tooth from being subjected to stains from both surfaces. After the teeth had been in the staining solutions for varying periods of time, some were bisected longitudinally, and others cut in cross-section blocks, and then ground approximately 10 $\mu$  on Black's grinding machine, under either water or paraffin oil. Great difficulty was encountered in preventing decolorization of the blocks during mounting, grinding, and demounting.

*f. Intra-vital Dyes.* Use of "intra-vital dyes" in studying formation and structure of enamel and dentine.

(1) *Madder Feeding.* Gottlieb<sup>32</sup> fed a series of dogs, from the beginning of tooth-eruption for three months, on madder. Ground sections made from the teeth of these animals showed that red-colored calcium had been deposited in dentine, the amelo-dentinal junction and enamel.

(2) *Sodium Alizarinsulphonate.* In the same report Gottlieb described an experiment on 2 dogs, 1 very young and 1 about a year and half old. They both were injected subcutaneously and intravenously with a 1 per cent solution of sodium alizarinsulphonate. Teeth taken from these dogs showed in the dentine adjacent to the pulp an intense violet coloration. Toward the periphery the intensity decreased but nevertheless the color did actually extend into the enamel.

<sup>31</sup> Leigh, R. W. *J. Am. Dent. Assoc.*, 12: 1415, 1925.

<sup>32</sup> Gottlieb, B. *Ztschr. f. Stomat.*, 11: 452, 1913.



(3) Trypan Blue. Similar work was carried on by Wellings.<sup>33</sup> Animals were injected with the maximum dose (1 cgm. to 20 gm. of body weight) of color solution at intervals of seven or eight days, until the desired degree of staining was reached, when they were killed, and the tissues instantaneously fixed by introducing 10 per cent formalin in normal saline through the heart. The solution of the dye was made in physiological circulating fluid, at first according to Ringer's formula, but later in Schiassi's fluid.<sup>34</sup> It was always boiled in a sterile test-tube before use. Injections were made under the strictest aseptic precautions. In the younger animals the entire head, without decalcification, was impregnated with gelatin according to Gaskell's methods.<sup>35</sup> In this method, the gelatin is formalinized to render it insoluble, after which fairly thin sections can be obtained by freezing the gelatin block with CO<sub>2</sub> and cutting on the Aschoff freezing microtome.

Older heads were decalcified with 5 per cent hydrochloric acid, with the addition of 1 per cent formalin to preserve the tissues. On the completion of decalcification the tissues were washed in tap water. Prolonged washing is undesirable. Then they were taken through the alcohol series (a little formalin added to all the dehydrating solutions prevents the washing out of the stain) into alcohol and ether, and thence into celloidin. In order to examine for finer cytological details it was necessary to imbed in paraffin. Here again all the dehydrating solutions should carry a little formalin with them. Ground sections were also prepared. In order to keep the section cool while grinding without decolorization dilute hydrochloric acid (1-1000) was found most satisfactory. Small fragments of soft tissues, i. e., pulp, were examined by teasing out in a drop of Farrant's solution.

By this method the matrix of dentine and bone is stained very lightly, the color disappearing quite soon. The walls of the dentinal tubules and the Haversian canals do not stain, neither do the dentinal fibrils. The secondary dentine, formed in the center of the pulp cavity of persistently growing teeth, stains deeply. Enamel developing at the time of the introduction of the stain, and that formed while the stain is in the body, takes on a very brilliant color which subsequently fades as decalcification progresses. It is not possible to stain already calcified enamel by means of trypan blue.

(4) Gies. Gies<sup>36</sup> gave a series of puppies occasional intraperitoneal injections of trypan blue, at different stages of the growth and dentition

<sup>33</sup> Wellings, A. W. *Trans. 6th Internat. Dent. Cong.*, Lond., 1914, p. 46.

<sup>34</sup> Schiassi, B. *La Semaine Méd.*, 1913.

<sup>35</sup> Gaskell, J. *Path. & Bacteriol.*, 17: 58, 1912.

<sup>36</sup> Gies, W. J. *J. Nat. Dent. Ass.*, 5: 529, 1918.

of the dogs, and found that when trypan blue had been injected, after the permanent teeth had attained their full size, the teeth did not take the stain. When the trypan blue had been injected after the development of the permanent teeth had been far advanced, but before attainment of full size, the teeth showed a narrow blue zone above the gum line, extending about one-fourth the distance to the occlusal surface. When the trypan blue had been injected, soon after the permanent teeth had begun to form, the teeth showed a wide blue zone extending about three-fourths of the distance from the gum line to the occlusal surface. When trypan blue was injected before the permanent teeth had begun to form, the teeth were blue over the entire surface of each crown.

In preparations from other dogs similarly treated the blue pigment was seen in both dentine and enamel.

(5) Marshall.<sup>37</sup> Marshall extended this work, using the following method:

The dyes which seemed to give the best results were naphthamine brilliant blue and trypan blue, employed in 1 per cent aqueous solutions and in 1 c.c. doses. The intervals between the injections were purposely arranged at one, two, three, four and five days. All solutions of the dyes, and the injections, were made under the strictest aseptic precautions. Immediately after killing the animal the head was placed in a glass container and covered with warm 10 per cent formalin solution. This was kept in an electric oven at a temperature of 35°C. for from three days to one week. Upon removal from the oven both jaws were dissected from the head, and each divided on the median lines, the parts again placed in warm aqueous solution of formalin, and returned to the oven at the previous temperature. Later, the parts were passed through the alcohols, from 40 per cent to absolute, increasing 5 per cent each successive twelve hours and then placed for from one to three weeks in benzol-damar, at room temperature. When the specimens were thoroughly impregnated with damar, they were transferred to the discs of a grinding machine, cemented in place with thicker benzol-damar, and again placed in an electric oven, this time at a temperature of 50°C., and allowed to remain for two or three days, or until the damar was sufficiently hardened to prevent the specimen from being torn from the disc in grinding.

By this method, Marshall demonstrated definite color lines in the dentine. Alizarin sodium sulphonate is the only red vital dye studied, that possesses the power to stain the dentine with definite color lines which can be seen under the microscope.

Schour of the University of Illinois College of Dentistry proposed a simpler method to obtain distinct red lines in the ground sections of vitally stained bone and teeth.

After sawing the specimen into small sections he found that routine

<sup>37</sup> Marshall, J. S. *J. Dent. Res.*, 3: 241, 1921.

grinding of the sections in a light grade of liquid petrolatum is sufficient and gives sharp results so that micrometer readings of the intervals between the injection lines are readily made.

(6) Schour.<sup>38</sup> Schour's experiments concerning vital staining with alizarin were undertaken for the purpose of studying normal processes of calcification and of making quantitative determination of the rate of formation of bone and dentine.

In order to determine the average amount of alveolar bone growth, he proceeded in the following manner: 16 white rats, three months of age, were given two intraperitoneal injections of 0.5 c.c. of 2 per cent alizarin red s (sodium sulphalizarate) of color index 1034 (Coleman and Bell Company) four or six days apart. The dosage ranged from 39 to 62 mg. per kg. The animals were sacrificed two days after the last injection.

Transversely ground sections were then prepared at the mid-root level of the upper molars which showed microscopically a red line in the alveolar bone where calcification took place at the time of each injection. The amount of bone laid down in twenty-four hours was obtained by measuring the distance between two red lines in micra and dividing the measured distance by the number of days intervening between the two injections. The measurements were made in areas where red lines followed a parallel course for at least a short distance.

Using this method the author was able to compare the daily apposition of alveolar bone to that of the daily apposition of enamel and dentine in the incisor of the rat.

The author believed that multiple injections of sodium sulphalizarate present advantages over feeding madder or alizarin and offer a prompt and accurate method of measuring the rate of apposition of bone in normal and experimental conditions.

Schour and Smith<sup>39</sup> presented a simple and supposedly accurate method for measuring the rate of growth of the enamel and dentine in continuously growing teeth. They used three groups of rats:

Group A: 12 rats were given two to eight injections of 0.3 c.c. of 2.5 per cent sodium fluoride twenty-four or twenty-eight hours apart. Age: ninety to 270 days.

Group B: 20 rats were given single injections of 0.3 c.c. of 2.5 per cent sodium fluoride and allowed to live one to forty-eight hours after the administration. Age: ninety to 270 days.

Group C: 16 control rats, most of which were littermate controls. Significant alterations were observed only in Groups A and B.

<sup>38</sup> Schour, I. *Proc. Soc. Exper. Biol. & Med.*, 34: 140-141, 1936.

<sup>39</sup> Schour, I., and Smith, M. C. *Proc. Soc. Exper. Biol. & Med.*, 32: 1, 1934.

(7) Trypan Blue, India Ink, Methylene Blue. Fish<sup>40</sup> injected intravenously or subcutaneously trypan blue and iron solutions for the study of dentine.

(8) India Ink. In a later experiment a young dog of a large breed was anesthetized and part of the crown of one of the canines was sawed off to expose the pulp. A syringe filled with India ink and fitted with the finest ordinary hypodermic needle was introduced along the wall of the pulp chamber to about half-way down the root, and while it was being slowly withdrawn the ink was allowed to leak out. No pressure whatever was used. After injection the pulp chamber was not sealed and the dog was killed after an hour or so. Then the tooth was cut in half and the ink was found to have penetrated the dentine. Sections were made to study the details of distribution of the carbon particles.

(9) Methylene Blue. In a continuation of this study Fish<sup>41</sup> anesthetized an adult dog, drilled a cavity in the crown of one of the teeth near the neck and freely exposed the pulp. A small quantity of solid methylene blue was introduced into the cavity and gently worked into the pulp with a probe, without destroying the tissues of the pulp. A trace of moisture was then added to the dye to ensure its solution in the lymph of the pulp and the cavity was covered with a smear of temporary cement. The dog was kept under morphia and was killed from twelve to twenty-four hours later. On examination a wide zone of a dense blue color was found around the part of the tooth at which the dye was introduced, and where it had entered, the enamel had the appearance of dark blue Bristol glass. Teeth so prepared may be preserved in 10 per cent formalin indefinitely and sections made by grinding. After they are ground the sections may be immersed in 6 per cent ammonium molybdate solution for twenty-four hours, then freshened up on the hone and mounted in Farrant's medium.

5. Examination of Ground Sections of Teeth by Polarized Light. Hoppe<sup>42</sup> examined ground sections of teeth by means of polarized light and renders the following account:

"The enamel shows on examination by polarized light a much greater degree of double refraction than the dentine and cementum. When the enamel prisms (or rods) are struck at right angles to their longitudinal axes and these axes are placed at an angle of  $45^{\circ}$  to the crossed nicols, the color differences are especially noticeable. As the enamel rods generally follow the spiral or curled course, the picture will offer a multicolored effect. Three or four colors may appear

<sup>40</sup> Fish, E. W. *Proc. Roy. Soc. Med., Sect. Odont.*, 19: 59, 1926.

<sup>41</sup> Fish, E. W. *Proc. Roy. Soc. Med., Sect. Odont.*, 20: 1, 1927.

<sup>42</sup> Hoppe, J. I. *Virchows Arch.*, 24: 29; 30; 31; 32, 1862.

simultaneously in the field of observation. It can be clearly noticed that the colors depend on the direction of the enamel rods.

"When no gypsum or glimmer plates are used and the nicols crossed, the transversely sectioned rods appear absolutely black, but when the prisms are parallel they appear light. At higher magnifications the difference in illumination is more noticeable. Developing enamel rods, which can be readily isolated, appear nearly as strongly double refracting as the fully formed enamel. On examination of different ground sections I invariably found the enamel negative when fully developed, positive when still in a formative state. Peculiar as this may seem, I always obtained the same results. The most remarkable fact however, is that after heating to 800°, the enamel again turns positive.

"The enamel consists almost entirely of inorganic substances. It may be assumed that double refraction is caused by the arrangement of mineral substances in the enamel. After heating to 800° the organic material was entirely destroyed and the double refraction reversed." (Translation.)

V. Ebner<sup>43</sup> stated that:

"The positive double refraction of developing enamel rods cannot be observed only in dry specimens but also in prisms which are examined in water or alcohol although the degree of positive double refraction is somewhat lessened. Many other fluids change the double refraction to a marked degree, turpentine, benzene and paraffin oil decrease the double refraction considerably, concentrated glycerin reduces it almost to 0° and causes sometimes a weak negative double refraction. Origanum oil, xylol and monobromnaphthalein act in a similar way. Anise oil and carbon disulphide act somewhat stronger. Cinnamon oil, cassia oil, oil of cloves, salicylic aldehyde and aniline oil cause a definitely negative double refraction, much weaker, however, than the original positive refraction. When, subsequently, enamel is washed with alcohol, it resumes the same degree of double refraction. We must, therefore, conclude that the fluids which so markedly change the optical behavior of the developing enamel produce no permanent structural changes in same. We may assume that just as stains, so the mentioned fluids are able to penetrate the apparently homogeneous rods but cause a change in tension in the minute pores of the rod substance which totally reverses the double refraction. The tensional changes are dependent on the absorptive qualities of permeability of the substance. The changes in double refraction so readily brought about in forming enamel rods appeared to me a definite proof of the assumption that during development the rod diameter not only increases in size but also that through impregnation of calcium salts the density of the enamel rods increases." (Translation.)

Viggo Andresen,<sup>44</sup> who quoted the mentioned authors, used polarized light to produce excellent photomicrographs which he obtained to sup-

<sup>43</sup> v. Ebner, V. *Ztschr. f. wiss. Mikr.*, 9: 161, 1892.

<sup>44</sup> Andresen, V., 1926 *The Physiological and Artificial Mineralization of Enamel*. Oslo, Dancke.

port his theory on the physiological and artificial mineralization of the enamel.

Kirk<sup>45</sup> applied polarized light in the examination of ground teeth but does not describe a method of interpretation.

**6. Examination of Ground and Decalcified Sections of Teeth by Ultraviolet Light.** Walkhoff<sup>46</sup> has applied ultraviolet light as a source of illumination for the microscopic examination of sections of teeth. Of course the lenses, slip and coverslip must be of quartz and the record is made on a photographic plate. Direct examination by the eye is impossible. Because of the shorter wave-length, this method possesses distinctly greater resolving power. Walkhoff was primarily interested in the problem of an interprismatic, cement-substance in the human enamel. The details of the technique are rather elaborate and are given in the original article.

Meyer<sup>47</sup> stimulated by Walkhoff's work, obtained excellent results with ultraviolet light in the study of ground sections of teeth. He used a wave length of 0.275 and a monochromat objective of 2.5 to show details of the enamel prisms and interprismatic substance, which are impossible to discern with the aid of ordinary light.

**7. Examination of Ground Sections of Teeth at Different Levels.** Making use of an objective with small focal depth, Meyer<sup>48</sup> was able to prove the statement of v. Ebner, namely that the so-called tufts of the enamel are poorly calcified sheaths of interprismatic substance. He first observed the surface of a thick enamel section and photographed it, then lowered the body tube of his microscope a trifle and photographed a slightly lower level of the enamel in which he could follow the disposition of the interprismatic defect. Subsequent exposures revealed the leaf-like nature of the tufts. His method finally yielded photomicrographs of different levels of the enamel in serial order, a result which could not be obtained by cutting and grinding.

**8. Examination of Ground Sections of Teeth by Roentgen Rays.**

(a) *The Diffraction Method* (a study of the crystal structure of the enamel and dentine). In 1926 two papers appeared<sup>49</sup> which promised a more refined analysis of the structure of enamel and dentine than has hitherto been possible. Laue's method of making roentgenphotograms was employed. The source of the roentgen rays was a Coolidge electron

<sup>45</sup> Kirk, E. C. *Dental Cosmos*, 45: 345, 1903.

Gerhardt. *Arch. f. Entwicklungsmechanik*, 10: 135, 263.

<sup>46</sup> Walkhoff, O. *Dental Cosmos*, 65: 65, 1923.

<sup>47</sup> Meyer, W. *Dtsch. Zahn. Mund-, u. Kieferhkl.*, 2: 43, 1935.

<sup>48</sup> Meyer's *Normal Histology of the Human Teeth and Associated Parts*, trans. and ed. by Herman R. Churchill. Phila., Lippincott, 1935.

<sup>49</sup> Funaoka, S. *Acta Scholae, med. univ. imp. Kyoto*, 9: 37: 41, 1926.

tube with a molybdenum anticathode. The radiation lasted from fifteen to fifty hours. The sections of the teeth were 1 to 0.3 mm. thick. In studying the dentine the directions of the rays were (a) at right angles to the dentinal fibers and (b) parallel with the dentinal fibers.

Cross, longitudinal and tangential sections of human incisors and molars of cattle were used in the study of the enamel. The sections were 1 to 0.4 mm. thick. They were so placed that a narrow bundle of roentgen rays would pass (a) parallel, (b) at right angles vertically, and (c) at right angles horizontally to the long axis of the enamel prism. The dentine had previously been ground away and the upper surface of the enamel section had been etched with dilute HCl in order to exclude the influence of the grinding on the arrangement of the superficial microcrystals.

Taylor and Sheard; Eisenberg; Cape and Kitchen; Roseberry; Hastings and Morse; Thewlis; Möller and Trömel; Bale, Hodge and Warren, and others published papers on this subject supplying new information on the crystalline constituents of enamel and dentine.<sup>50</sup>

(b) *The Absorption Method*.<sup>51</sup> (a study of the changes in the enamel and the dentine by quantitative measurements of the film densities obtained by exposing sections of teeth to roentgen rays). Price<sup>52</sup> compared semi-quantitatively the opacities of the hard tissues of teeth with the opacities of different steps of a simultaneously exposed aluminum or copper penetrometer.

Boedeker and Applebaum<sup>53</sup> used a penetrometer of aluminum to express semi-quantitatively the differences found in a carious section of a tooth of unmeasured thickness.

Thewlis<sup>54</sup> compared the total absorption of the roentgen rays by enamel and dentine in a human and a dog's tooth.

Again Boedeker and Applebaum<sup>55</sup> showed changes in the dentine

<sup>50</sup> Taylor, N. W., and Sheard, C. *J. Biol. Chem.*, 81: 479, 1929.

Eisenberg, M. J. *Am. Dent. Surgeon*, 50: 231, 1930. *Dental Cosmos*, 75: 770, 1933.

Cape, A. T., and Kitchen, P. C. *J. Am. Dent. Assoc.*, 17: 193, 1930.

Roseberry, H. H., Hastings, A. B., and Morse, J. K. *J. Biol. Chem.*, 90: 395, 1931.

Thewlis, J. *Brit. J. Rad.*, 5: 353, 1932.

Möller, H., and Trömel, G. *Naturwiss.*, 21: 346, 1933.

Bale, W. F., Hodge, H. C., and Warren, S. L. *Am. J. Roentg. & Rad. Ther.*, 32: 369, 1934.

<sup>51</sup> The following information was taken from Van Huysen, G., Hodge, H. C., Warren, S. L., and Bishop, F. W. Quantitative roentgen ray study of certain pathological changes in dentine. *Dental Cosmos*, 75: 729-738, 1933.

<sup>52</sup> Price, W. A. *Dental Cosmos*, 42: 117, 1900.

<sup>53</sup> Boedeker, C. F., and Applebaum, E. *Dental Cosmos*, 73: 995, 1931.

<sup>54</sup> Thewlis. *Loc. cit.*

<sup>55</sup> Boedeker, C. F., and Applebaum, E. *Dental Cosmos*, 75: 21, 1933.

surrounding dental caries by means of roentgen-ray films. They compared qualitatively areas of dentine of different opacities in the film with corresponding areas from the specimen.

Cahn<sup>56</sup> showed radiolucency in the root of a whole tooth as evidence of change in dentine.

Heiwinkel<sup>57</sup> studied the appearance of twenty-one 1 mm. thick tooth sections using reflected and transmitted light and roentgen rays, and noted a congruence in the pictures by transmitted light to the pictures by roentgen rays. Transparent areas in the dentine associated with caries were shown to be relatively radiopaque in some of the cases.

Van Huysen, Hodge, Warren and Bishop<sup>58</sup> described a method of studying the changes in dentine by taking precise measurements of roentgen-ray opacities of different areas. They also studied the variations in the roentgen-ray opacity of the dentine of certain teeth affected by caries. They adopted the following procedure:

Three sections about 1 mm. thick were sliced near the center of a carious area of the tooth and then surface ground, plano-parallel with a variation not greater than about 0.1 mm. Two penetrometers were made of cast aluminum, which were experimentally shown to have the same coefficient of absorption of roentgen rays as the dentine.

They were 15 mm. wide, 4 cm. long and were provided with eight steps, each 5 mm. wide. The thickness of the first step was 2 mm., the following steps each 0.25 less, so that the last step presented a thickness of 0.25 mm. The maximum variation of each step was about 0.1 mm.

The three sections were arranged in a vertical row and flanked by a penetrometer at each side of the sections. Two exposures were then made on the left and right half of a 5 x 7 inch roentgen-ray film wrapped in black carbon paper. The exposures were made with a 30 milliamperere radiator-type Coolidge tube, controlled by a Waite and Bartlett oil-emersed single valve, one-half wave rectified equipment. Each exposure was made at 30 kilovolts, 28 milliamperes, 2.5 seconds, with the target centered over the two sections 15 inches from the surface of the film. No filtration was used.

After developing and drying, the density of the silver deposits in the film was obtained by means of the Capstaff-Purdy densitometer built by the Eastman Kodak Company. This instruments measures a 0.5 mm.<sup>2</sup> area of film and reads directly in density units.

The average of 4 density readings on the film image of each step of the penetrometer were then plotted against the thickness in millimeters of aluminum. Thus was obtained an absorption curve for the film used (density-thickness curve). A density reading in the dentine could now, with the aid of the density-

<sup>56</sup> Cahn, L. R. *Dental Cosmos*, 74: 1164, 1932.

<sup>57</sup> Heiwinkel. *Vierteljahrsch. f. Zahnhk.*, 48: Pt. 2, 247, 1932.

<sup>58</sup> Van Huysen, G., Hodge, H. C., Warren, S. L., and Bishop, F. W. *Dental Cosmos*, 75: 729, 1933.



thickness curve, be readily interpolated into density value in terms of millimeters of aluminum.

The method of Van Huysen et al. offsets the qualitative nature of radiopacity as ordinarily observed in photographic films. Qualitative determinations obviously limit the value as a criterion of density of calcification for experimental purposes.

They stated that it is possible to duplicate these results with a total error of less than 5 per cent.

Hollander<sup>59</sup> believed that the error in the foregoing method must be greater since they used a mean thickness value throughout the section, but did not include its precision measure in that of the entire determination. He stated:

"Our experience<sup>60</sup> with *grenz-ray* radiography of ground sections of teeth suggested that differences of considerably less than 5 per cent may be significant in studies of normal calcification. In order to measure such differences, a method has been developed for the measurement of radiopacity which is good to 0.5 per cent or better. This method employs soft x-rays and is based on the principle of the double ionization chamber described by Becker<sup>61</sup> for measuring minute changes in x-ray intensity. The apparatus was changed completely, however, in order to adapt it to the special requirements of a routine dental investigation. It consists of two symmetrical ionization chambers with a common collecting plate and individual charged plates at equal but opposite potential. Since the charge on the collecting plate represents the difference between the electrical effects in the two chambers, a string electrometer is used as a null instrument. The chambers are so placed over the *grenz-ray* tube that the radiation strikes their apertures symmetrically. A specially constructed mechanical stage, provided with coordinate scales in the usual way, supports the specimen directly under one aperture; under the other, which has the same diameter, there are placed standards of measurement made of aluminum foil. Thus the size of specimen on which measurements are made is determined by the aperture. A micrometric arrangement for varying the length of air column permits of interpolation. Before starting measurements, the mechanical stage is placed on a microscope and areas, designated by their coordinate scale readings, are chosen for study. These circular areas are recorded on a photomicrograph of the specimen. To determine thickness at each position, provision is made for cutting out each disc and measuring it with a micrometer caliper.

The resulting apparatus is essentially a comparator whereby a minute area of ground section can be compared with a series of calibrated standards until two are found which most nearly match the unknown. Interpolation between

<sup>59</sup> Hollander, F. *Proc. Soc. Exper. Biol. & Med.*, 33: 388, 1935.

<sup>60</sup> Hollander, F., Applebaum, E., and Boedecker, C. F. *Proc. Soc. Exper. Biol. & Med.*, 30: 1315, 1933.

<sup>61</sup> Becker, J. A. *Physical Rev.*, 20: 134, 1922.

these two values is effected by means of the air column scales. Measurements of the absorption coefficients are relative, being expressed as number of micra of aluminum which possess the same absorptive capacity. The precision of the method is indicated by the following: In a series of duplicate determinations on sixty different areas, the S.D. was  $\pm 0.2$  per cent or less in all but four instances. Also, for a micrometer caliper reading of  $300\mu$  the S.D. was almost never greater than  $\pm 0.3$  per cent. Consequently the precision measure of the entire determination is around  $\pm 0.5$  per cent for circa  $0.25 \text{ mm.}^2$  ( $0.7 \text{ mg.}$ ) of tooth substance. Measurements on non-carious human teeth have already shown small but characteristic differences within individual sections as well as striking similarities among different teeth. For an experimental study involving animals with smaller teeth, it is necessary only that the aperture size be reduced to  $0.5 \text{ mm.}$ "

In a recent paper Hollander and Vesely<sup>62</sup> reported "x-ray absorption coefficients of coronal and root dentine," obtained with the precision micromethod described in Hollander's article (see foregoing).

9. Examination of Ground Sections of Teeth by Reflected Light (Opaque Illumination). A type of microscope often employed in mineralogical studies is used.<sup>63</sup> It is important to have a brilliant source of illumination, as an arc light, exactly centered to cast its beam horizontally through a lateral opening in the tube of the microscope onto a prism. This prism lies within the tube and is adjusted at such an angle that the light is thrown down onto the object. The light is reflected therefrom back up the tube to the eye of the examiner. An advantage of this method of examination is that it renders unnecessary the tedious preparation of thin ground sections. It is, however, absolutely necessary that the surface of the tooth be very highly polished and be placed parallel to the stage of the microscope. Beautiful photomicrographs, even at a magnification of 1600 diameters, have been made in this way.

A somewhat similar method has been devised by Köhler and Sonnenburg<sup>64</sup> which, however, does not require the special mineralogical or metallurgical microscope.

(1) The tooth to be examined may be fresh or fixed. It may have been subjected to vital or supravital staining, or it may have been prepared by the Koch-Weil petrification method. Sometimes it is desirable in order to prevent too deep a penetration of the stain after grinding, to cover it with a thin layer of some transparent material, as an acetone-celloidin solution.

(2) The tooth is then set in a plaster-of-Paris base, whose under surface determines—is parallel to—the plane of grinding. Enough of the tooth should project above this base to permit the focusing of a beam of light within the body of the tooth. The base is conveniently prepared as follows: Cut a section of

<sup>62</sup> Hollander, F., and Vesely, E. *Proc. Soc. Exper. Biol. & Med.*, 34: 158, 1936.

<sup>63</sup> Friedeberg. *Deutsche Monatschr. f. Zahnk.*, 40: 57, 1922.

<sup>64</sup> Köhler and Sonnenburg. *Vrtljhrshr. f. Zahnk.*, 29: 230, 1923.

rubber tubing, 1 inch or more in diameter and as long as the tooth in question. Mix a thin batter of plaster and pour it into the tubing which rests on a glass slab. The plaster should come up about half way in the tubing. The tooth should be inserted into the plaster in such a manner that the plane of the glass slab is parallel to the plane through the tooth, which it is desired to examine.

(3) The tooth is held against a revolving grinding stone and ground down to the plane which it is desired to examine. It is most essential that the base be held in a plane parallel to the stone. This can be easily done by the use of various mechanical devices.

(4) The ground surface is carefully and thoroughly polished on an Arkansas stone with aluminum oxide.

(5) This surface is etched five, ten or twenty minutes with 6 per cent nitric acid, which is washed off with water.

(6) The acid is neutralized by ammonia vapor. The block with the tooth is placed on a piece of blotting paper moistened with ammonia water and covered with a small belljar. One can now immediately pass to (10) below or he can apply a stain.

(7) Apply Delafield's hematoxylin to ground surface, two to five minutes.

(8) Differentiate in distilled water to which a little acid is added (*ca.* 1:20).

(9) Repeat (6). A shorter time will suffice.

(10) Wash in increasing concentrations of alcohol, then carbol-xylol, and attach a cover glass to the ground surface with Canada balsam. It is sometimes desirable, especially if high magnification be desired, to clear the entire tooth in glycerin or by the Spalteholz method before attaching the cover glass.

After the balsam has hardened, the plaster base is broken off and the tooth is suspended by the cover glass into a small glass cylinder.

The specimen is examined by the following manner of illumination: Horizontal rays from a carbon arc are filtered through water, and collected by a bi-convex lens which focuses them to a point within the substance of the tooth. This point lies in the optical axis of the microscope and below the ground surface to be examined. This ground surface forms an optical plane or section which is examined in the usual way by the microscope.

Any number of such optical planes in a given tooth may be studied and photographically recorded, by simply removing the cover glass and grinding down to a new surface and repeating (4) to (10) of this method.

**10. Interglobular Spaces.** These can usually be seen in unstained ground sections of teeth, particularly when they have been dried before mounting in balsam. However they can be made more conspicuous in at least three ways:

(1) When the ground section is 0.5 mm. or slightly less in thickness, let it stand overnight in the dark in a 1 per cent aqueous solution of silver nitrate. Remove, rinse in distilled water, and then immerse in 1 per cent formalin in

direct sunlight until the specimen becomes deep black. Then finish grinding to desired thickness, polish and eventually mount in balsam.

(2) Stain ground sections in a hematoxylin solution for a few minutes, partially decolorize with very dilute acetic acid. Control this under the microscope. Rinse in distilled water as soon as only the interglobular spaces retain their color: then into 1 per cent osmium tetroxide (osmic acid) for one hour. Wash, clear and mount.

(3) (*Charters White's Method.*) Sections of teeth, *ca.* 1 mm. thick, are dehydrated, passed through ether and absolute alcohol (equal parts), and then into a stained celloidin solution for several days. The celloidin solution is made as follows: add fuchsin to absolute alcohol until a "dark port wine color is produced," mix this with an equal part of ether and add celloidin to required consistency. Remove sections and dry by evaporation. They may be kept indefinitely in this condition. When desired they are ground to the required thickness, polished and mounted in balsam.

**11. Lymphatics of Pulp.** *a. Schweitzer*<sup>65</sup> injected the blood vessels with a carmine mass and the lymph vessels with Berlin blue-turpentine-ether mixture, according to the method of Gerota.<sup>66</sup>

The animals were either killed by chloroform and immediately injected; or after rigor had passed were warmed to about 50°C. The injection was made into the common carotid or in the case of the smaller animals into the ascending aorta after opening a vein. The pressure must be maintained for some time in order to insure the injection of the gingival capillaries as indicated by the reddening of the mucosa. The lymph vessels were injected by the same method as that used for macroscopic studies, only with great care not to use too strong pressures. Schweitzer assumed that the lymph vessels of the pulp, if such exist, probably communicate by anastomosis with those of the parodontal tissue. Therefore, by injecting the lymph vessels of the gingiva near the teeth, it could be expected that under favorable conditions one might force the fluid from these into the lymphatics of the pulp, regardless of a flow in a retrograde direction. In this way Schweitzer actually succeeded in filling lymph vessels in the pulp in some cases. Schweitzer's experience leads him to a conclusion that when the purpose of the studies is to reveal the distribution of the lymph vessels, it is desirable to have the injection of the lymph vessels precede that of the blood vascular system. The tissues were fixed and hardened in formalin (alcohol or Müller's fluid, diluted 1:10), decalcified in 1 per cent hydrochloric acid, imbedded in parlodion and serially cut at 200 $\mu$ .

*b. Dewey and Noyes.* The work of Dewey and Noyes<sup>67</sup> was done

<sup>65</sup> Schweitzer, G. *Arch. f. mikr. Anat.*, 74: 927, 1909.

<sup>66</sup> Gerota. *Anat. Anz.*, 12: 216, 1896.

<sup>67</sup> Dewey, K., and Noyes, F. *Dental Cosmos*, 59: 436, 1917.

chiefly on dogs. In all instances the blood vessels were injected with carmine gelatin in order to eliminate confusion as to whether the channels were lymph vessels, or arteries and veins. After ligation of the superior vena cava and the arch of the aorta in two places and opening the jugular vein or the vena cava, the cannula was inserted into the left common carotid. The vessels were thoroughly washed out with physiological salt solution and the carmine gelatin mass injected. The body was cooled off under running water for from five to ten minutes. Before beginning with the injection of the lymph vessels, some time was allowed to pass, to insure the complete solidification of the gelatin. The head was severed, the fur removed, and the head warmed in warm water. They used the injecting mass which is now most frequently employed, i. e. the Gerota mass. For this, 2 gm. Prussian blue (oil color in tubes) is stirred with 3 gm. turpentine oil in a glass mortar for five minutes, 15 gm. of sulphuric ether is added and this fluid is filtered through flannel or chamois skin. They used ordinary fine steel needles with Luer's syringe. This facilitated keeping the injecting point as near the deep portions of the teeth as desired. While injecting, slight pressure was made with the finger over the place of the inserted needle, and after withdrawing the latter, gentle even massage was continued for some time. All excess of the fluid on the external surface was carefully washed off. The head was left on the table for an hour or longer, and thereupon placed in 20 per cent formalin. After twenty-four hours, or at any later period, the injected teeth were singly sawed out from the jaw, carefully opened, and the pulp removed and examined.

Another method for injecting the lymphatics of the dental pulp was used by Dewey and Noyes and called by them "the direct method." Because the individual injection of a number of teeth consumes considerable time, that of the blood vessels was made first. A hole was made in the tooth, reaching down to the pulp, and a metal cannula made for this purpose was inserted. Injections were also made with Luer's syringe and a steel needle, the point of which had been filed off to the base of the needle. Both the metal cannula and the syringe barrel were filled with a few cubic centimeters of Prussian blue by means of a medicine dropper. Pressure was exerted in the syringe with the piston over a column of air, in the metal cannula, through a pressure tank or by blowing with the mouth for longer or shorter periods into paper cones, the tapered ends of which were inserted into the cannula. Unless the needle or the cannula fits absolutely into the hole the fluid invariably flows back, at even very slight pressure. Special cannulas were made with a conical point to fit tightly in a hole made by a drill with a special thread; with these there was no backward flow. In using the pressure

tank, jerky movements produced by compressing the rubber tube in quick succession seemed to be more effective than an even pressure.

Finally, the order of procedure was reversed, and the injection of Prussian blue was made before the blood vessels were filled and distended with carmine gelatin, as it was evidently this condition which rendered the tissues so unduly resistant. The heads and necks of freshly killed dogs were kept warm by constantly applying hot wet cloths to them. Pressure was again effected by blowing or by the air-tank so applied as to bring about the above-mentioned jerky movements. The injection of the blood vessels followed some time later. The results obtained with these procedures, although applied only on a few dogs, were positive in every case, and far superior to any of the previous ones. The blue injection mass had filled lymph vessels emerging from the maxilla and the mandible and passed in considerable quantities into the sub-maxillary lymph glands and deep cervical glands.

Supplementary studies of the lymphatics of the pulp were made by an entirely different manner of investigation. A large number of rabbits and also a few dogs and cats were injected intravenously or intraperitoneally with trypan blue or lithium carmine for various periods of time. In these, the blood vessels of the head were well injected with carmine or Berlin-blue gelatin, after which the tissue was put into 10 per cent formalin. After hardening, the pulps of the incisors were removed and frozen sections were made. This method gave suggestive but not conclusive evidence that lymphatics are present in the dental pulp.

**12. Innervation of Pulp, Dentine and Dental Periosteum.** *a.* Huber<sup>68</sup> used "intra-vitam" staining with methylene blue to differentiate myelinic and ammyelinic nerves in the pulp of the rabbit. Kill the animal with chloroform, insert cannula into common carotid, inject enough 1 per cent methylene blue in physiologic sodium chloride solution to pigment deeply tongue and lips. In half an hour remove mandible and wipe with a clean dry cloth. Remove molar tooth with aid of bone-forceps. Cut away dentine on anterior and posterior surfaces. Insert needle under one of the processes of the pulp and with slight traction carefully remove pulp with as little laceration as possible. Place pulp on slip moistened with physiologic salt solution. In a few moments the axis cylinders are found to be stained deep blue. For permanent preparations fix with a saturated aqueous solution of ammonium picrate or ammonium molybdate: then mount in glycerin. Sections can be cut by placing the pulp between 2 flat pieces of elderpith.

*b.* Dependorf<sup>69</sup> used, successfully in his opinion, the methods of

<sup>68</sup> Huber, C. *Dental Cosmos*, p. 84, 1898.

<sup>69</sup> Dependorf, T. *Deutsche Monatschr. f. Zahnh.*, 31: 377, 1913.

Loewit, Bielschowsky and Held to demonstrate the entrance of nerves from the pulp into the dentine and their course in this tissue.

He also studied the distribution of the nerves in the human alveolo-dental periosteum.<sup>70</sup> His methods, given in detail, were applied to teeth immediately after removal from the body and cut into small pieces. Different staining methods were employed: (1) the Cajal method, (2) Bielschowsky method, (3) the Golgi method, (4) the Loewit method. A modification of the Loewit method gave the most beautiful results. The details of these methods as used by Dendorff follow:

- (1) Cajal Method. (a) Place small pieces in 1.5 to 3 per cent silver nitrate solution at 37°C. and keep in the incubator at 37°C. for from three to five days.
- (b) Rinse quickly in distilled water at 37°C.
- (c) Reduce in pyrogallol formalin solution.
- (d) Frozen sections may be made, or, in case of calcified sections, decalcify in 5 to 10 per cent formic acid.
- (e) Imbed in celloidin and eventually, after sectioning, stain with the Knal silver solution (ammoniacal silver solution) according to Bielschowsky (thirty to fifty minutes).

Small pieces can also after preliminary staining with silver nitrate be quickly washed and then counterstained with the Knal silver solution for one to two hours at 37°C.

- (2) Bielschowsky Method. (a) Fix small pieces in 10 per cent formalin up to fourteen days or in 60 per cent alcohol plus 40 per cent formalin, 90:10.
- (b) Wash for from one to one and one-half hours in distilled water.
- (c) Cut frozen sections. The smaller pieces may also be stained further in toto and later cut.
- (d) Wash in distilled water, two to eight hours.
- (e) Immerse in 2 per cent silver nitrate for twenty-four to forty-eight hours or in the case of larger pieces up to four days.
- (f) Wash in distilled water, twenty-four hours.
- (g) Stain in the Knal silver solution according to Bielschowsky, thirty to sixty minutes; in the case of larger pieces, two to three hours at 37°C.
- (h) Immerse momentarily in 10 c.c. of distilled water plus 2 drops of glacial acetic acid. Rinse in distilled water.
- (i) Reduce in 20 per cent formalin solution with tap water, for twenty-four hours, and eventually counterstain by gold.
- (3) Golgi Method. (a) Fix small pieces in 8 parts of a 2 per cent potassium bichromate solution plus 1 part of a 1 per cent omic acid solution.
- (b) After five days rinse in distilled water or in 1 per cent silver solution.
- (c) Immerse in a large volume of 1 per cent silver solution for six to fourteen days and keep in semi-darkness.
- (d) Absolute or 96 per cent alcohol, one-half to one hour.

<sup>70</sup> *Deutsche Monatschr. f. Zahnh.*, 31: 853, 1913.

(e) Counterstain in 1 per cent gold chloride solution (8 to 10 drops in 10 c.c. of absolute alcohol) one-half to two hours.

(f) Rinse briefly in 50 per cent alcohol and in distilled water.

(g) Carry over into 10 per cent sodium sulphite solution (five to fifteen minutes).

(h) Wash for a prolonged period and counterstain.

(4) Loewit Method. (a) Fix in formic acid solution (1 part formic acid, 2 parts distilled water), five to ten minutes.

(b) Immerse in 0.25 to 1 per cent gold chloride solution from a few hours to one-half a day in the dark.

(c) Carry over into formic acid solution of strength above mentioned in (a), and leave for twenty-four hours in the dark.

(d) Reduce in concentrated formic solution (twenty-four hours in the dark).

An alternative procedure is (a) fix at 37°C.; (b) wash quickly in distilled water at 37°C.; (c) immerse in gold chloride solution (10 parts of distilled water, 2 parts of 1 per cent gold chloride) for two to six hours at 37°C., (d) wash briefly at 37°C., (e) immerse in the formic acid solution, of the strength mentioned above in (a), for two days at 37°C.; change once, keep in the dark. (f) Reduce and decalcify in concentrated formic acid for two to three days in the cold and in the dark.

c. *Fritsch*<sup>71</sup> also studied the innervation of the dentine. The method is as follows:

(1) Fix in formalin four weeks at least.

(2) Decalcify, Schaeffer's method.

(3) Cut frozen sections, Reich's method.

(4) Stain in methylene blue, Muench's method, or better.

(5) After Bielschowsky's method keep sections twenty-four hours in water.

(6) Treat with pyridine three to four days.

(7) Wash in distilled water, twenty-four hours.

(8) Treat with 3 to 5 per cent silver nitrate, five to eight days in the dark.

(9) Transfer to a silver oxide bath for four to five minutes.

(10) Treat with 20 per cent formalin for reduction.

(11) Wash with acidified distilled water.

(12) Wash thoroughly with distilled water.

(13) Repeat the steps 9 to 12, best results coming from 10 to 12 repetitions.

(14) Tone sections with gold.

(15) Cover with gelatin on a slip by transferring from water to a solution having 10 gm. gelatin to 100 c.c. water.

(16) Dry gelatin and examine sections under oil immersion lens.

d. *Tojoda*<sup>72</sup> approached the problem of dentine innervation from a new angle. Using conventional stains he noticed phenomena in his

<sup>71</sup> Fritsch, C. *Arch. f. mikr. Anat.*, 84: 307, 1914.

<sup>72</sup> Tojoda's method is published by Dieck, W. *Korr. f. Zahnärzte*, 51: 138, 1927.



specimens suggestive of Liesegang's rings. He suspected that these conditions were due either to a diffusion of silver nitrate in the stain through the gel of the dentine matrix or to the concretionary structure of the calcospherites. Zocher of the Kaiser Wilhelm Institute of Physical and Electrical Chemistry showed that when the dentine gel was freed of the chlorine ions by electro-osmosis the rings did not appear. Then the specimens were cleared of the precipitate resulting from the presence of a minute fraction of acid and hydrated lime salts. This precipitate is said to envelop the nervous tissue, preventing it from taking the stain.

The time of the electro-osmotic cleansing process is proportional to the size of the specimen. During this process the hue of the specimen gradually changes to a dead white in a direction from the cathode to the anode. When this change is completed the treatment is finished. The distilled water used must be changed frequently to avoid prolonging the process.

Washing for twenty-four hours does not seem to remove the residual acid from the specimen. When after washing for twenty-four hours the specimen was placed in a tube with distilled water and the electric current passed through, the tube was heated as a result of lowered conductivity through the presence of free acid. When after washing two or three hours the specimen was placed in lithium carbonate, then washed for twenty-four hours and treated by electro-osmosis, no increase in temperature was noticed.

The specimens are fixed in 10 per cent solution of formic acid reduced with a 10 per cent solution of formalin; then one proceeds to concentrate gradually.

Bielschowsky's stain seems to give the best results. Staining in block is preferred over staining in section.

*e. Berkelbach van der Sprenkel*<sup>73</sup> used the following technique:

"The dentinal wall of normal, healthy canini is ground until 300-500 $\mu$  in thickness. This leaves the pulp untouched and the cavity closed. Subsequently, the remaining part of the tooth is sawn into rings (not decalcified), and from these rings I made cross sections of about 40 $\mu$  in thickness on the freezing microtome. These are then impregnated according to the Gros method, in which naturally, the normal undecalcified dentine absorbs the silver very avidly. Though not elegant this way proved to be the most efficient."

The same author studied the innervation of the dental periosteum in many horizontally cut series from the mandible of young mice according to the Castro-Somnifen technique.

<sup>73</sup> Berkelbach van der Sprenkel, H. B. *J. Anat.*, 70: 233, 1936.

13. Form and Ramifications of Pulp Chamber and Canals. *a.* The work of Preiswerk<sup>74</sup> aroused interest in this subject.

Open the teeth from the occlusal surface into the pulp chamber: put in water for two or three weeks at about 37°C., wash through with hot soda solution, slowly dry in hot air and surround the roots with blotting paper (to prevent plaster entering apical foramen). Imbed in plaster of Paris and insert into each tooth, with white glue, a high cardboard funnel. Carefully warm for a day over a sand or water bath, then slowly raise temperature until Wood's metal in an adjacent dish begins to melt. Then pour molten Wood's metal abundantly into the funnel to secure enough pressure. Gently tap the preparation against the table to help the downward flow of the metal. After cooling, remove plaster of Paris and saw away the excess metal projecting from the tooth. If the metal comes through the tooth and lies between the apical end and the blotting paper, good results may be expected.

Put the specimen for two to three weeks in *ca.* 20 per cent KOH in an incubator, after which the metal core can be easily shelled out. Dry, varnish with very thin Canada balsam and mount.

*b. Fischer*<sup>75</sup> substituted celluloid for the metal casting of Preiswerk. The teeth were kept for several weeks in water at 37°C., to macerate the pulps. They were then gradually transferred to acetone solutions and finally injected with celluloid-acetone solutions of increasing concentrations. After complete hardening (evaporation of the celluloid solution) the injected teeth were macerated in pure HCl until every trace of organic or inorganic tissue had disappeared.

*c. Adloff.* Another method has been employed by Adloff.<sup>76</sup> The teeth were first decalcified, then bleached in hydrogen peroxide by adding it to the decalcifying solution, dehydrated, and cleared in cedar-oil. Permanent preparations can be made by immersing the specimens in vials filled with Canada balsam (not xylol- or chloroform-balsam).

This method is applicable to teeth whose canals have been filled with Wood's metal to demonstrate ramifications of pulp. The teeth, after they have been thoroughly cleared of pulp, decalcified and bleached, are invested in a casting-compound and the molten metal forced in by centrifugal force (tooth and investment at room temperature). They should then be clarified.

*d. Fasoli and Arlotta*<sup>77</sup> followed a slightly different procedure. They forced Wood's metal into empty canals of teeth. Some of these specimens were then radiographed while others were placed in a clearing solution (Spalteholz).

<sup>74</sup> Preiswerk, G. *Österr.-ungar. Vrtljhrschr. f. Zahnh.*, 17: 145, 1901.

<sup>75</sup> Fischer, G. *Deutsche Monatschr. f. Zahnh.*, 25: 544, 1907.

<sup>76</sup> Adloff, P. *Deutsche Monatschr. f. Zahnh.*, 31: 445, 1913.

<sup>77</sup> Fasoli, G., and Arlotta, A. *La Stomatologia*, 11: 409, 1913.

e. Moral<sup>78</sup> opened wide the pulp chamber of the tooth, which was then macerated and briefly bleached in hydrogen peroxide. It was then washed and dried. China ink was introduced after examination with a lens showed freedom from cracks. A fine smooth broach was used to introduce the ink as deeply as possible. Then the teeth were fixed upright on a glass plate and set in a moist chamber. The apices were directed downwards so that the ink of its own weight would tend to penetrate more deeply. When the ink came through to the apex (this usually took twenty-four hours) the tooth was removed from the moist chamber, dried, and after two to three days placed in 15 per cent nitric acid for decalcification. Hydrogen peroxide was added to decalcifying fluid for bleaching and the specimen clarified following Krause's method.<sup>79</sup>

f. Hess and Zürcher<sup>80</sup> have studied this field very thoroughly. Extracted teeth are drilled into and the pulp cavity is exposed, without however penetrating into the root-canals. Then the teeth are placed in a vessel filled with drinking water, and kept from three to four months in the thermostat at a temperature of 37°C., until the canals can be syringed through from the pulp chamber with a solution of soda at a temperature of 40°C. This result might be obtained in half the time if hydrogen peroxide, free from acid, were used. The teeth are washed for twenty-four hours in absolute alcohol, and are then dried for twenty-four hours at the temperature of a living-room.

Then the roots of the teeth are wrapped in blotting paper to the enamel margin, and they are imbedded in plaster of Paris in an ordinary well-fitting vulcanizing flask, so that the plaster of Paris covers the tooth up to the pulp cavity. When the plaster of Paris has hardened, the pulp cavity of each tooth is packed with rubber, the surface rubbed with talc, and the counter-mould made.

After the hardening of the plaster, the flask is opened and the root-canals and the pulp chambers of the teeth are packed as firmly as possible with ordinary red vulcanite. To avoid adherence to the counter-mould a piece of linen is placed between the two halves. The flask is now put into warm water and boiled for about twenty minutes, and afterwards closed by gradually increasing pressure. When the rubber has penetrated into the root-canals from the pulp chamber, another piece of rubber is put over each pulp cavity, and the flask is then boiled for twenty minutes and again closed by gradual pressure. The flask is then

<sup>78</sup> Moral, H. *Deutsche Monatschr. f. Zahnh.*, 32: 617, 1914.

<sup>79</sup> Krause, R. *Anat. Anz.*, 34: 133.

<sup>80</sup> Hess, W., and Zürcher, E. *Anatomy of the Root Canals of the Teeth*. Lond., 1925.

kept for several hours under the press, gradually cooled, and then during one hour vulcanized under a pressure of 7 atmospheres.

When removed from the vulcanizer, and after cooling the flask, the teeth can be removed from the plaster of Paris with the usual precautions, as now the plaster of Paris is worked easily; or, if not yet soft enough, the flask should stand in water for some days, to soften the plaster of Paris.

After the teeth have been rinsed in water, they are dissolved in a solution of 50 per cent pure hydrochloric acid, which can be done in the thermostat at a temperature of 25°C. to 31°C. in a few hours. The corrosion preparation is now rinsed carefully in running water, freed from the excess of vulcanite, and planted in a plaster of Paris block to form permanent preparations.

g. Barrett<sup>81</sup> has confirmed and extended these observations by simply following the dental pulp through a series of celloidin sections, stained with hematoxylin and eosin, of human teeth, cut approximately parallel with the occlusal surface.

14. **Innervation of the Gingiva.** Mowry,<sup>82</sup> who studied the nervous supply of the gingiva, obtained results by two methods of staining: one a modification of the Ranvier and Cohnheim gold chloride method (a) and the other a modification of the Bielschowsky silver nitrate method (b). Besides using selective stains he advises the use of fairly thick sections (15 $\mu$  or more) and the tracing of the fiber through changing foci.

(a) *Ranvier and Cohnheim gold chloride method (modified):*

(1) A piece of fresh tissue is fixed for at least forty-eight hours, in 10 per cent neutral formalin (40 per cent solution of gaseous formaldehyde).

(2) The tissue is washed in distilled water for fifteen minutes, and frozen sections (15 $\mu$ ) are cut and floated on distilled water.

(3) The selected sections are placed in a small dish of freshly expressed and filtered juice of a lemon and left to be mordanted for ten minutes.

(4) The sections are washed rapidly in distilled water and stained with a fresh 1 per cent solution of gold chloride for one hour.

(5) The sections are then transferred directly to a large quantity of acidulated water (10 c.c. of acetic acid to 250 c.c. of distilled water), and left for twenty-four hours, during which time the nerves become apparent.

(6) The sections are then washed in water and differentiated for thirty seconds in 50 c.c. of a freshly prepared solution of 5 per cent sodium thiosulphate.

(7) They are then washed for two minutes in distilled water and counter-stained rapidly by dipping, and removed immediately from a concentrated solution of picric acid.

<sup>81</sup> Barrett, M. T. *Dental Cosmos*, 67: 581, 1925.

<sup>82</sup> Mowry, D. P. Innervation of the gingiva. *J. Am. Dent. Assoc.*, 17: 1050, 1930.

(8) The sections are washed well, and rapid but thorough dehydration follows. They are then cleared in xylene and mounted in Canada balsam.

(9) The nerve fibers are stained a dark purple, while other tissue is yellowish.

*(b) Bielschowsky silver nitrate stain (modified).<sup>83</sup>*

(1) A piece of fresh tissue, not exceeding 1 mm. in thickness, is fixed for at least forty-eight hours in 10 per cent formalin.

(2) The tissue is washed in distilled water for fifteen minutes and thin frozen sections are cut and floated on distilled water.

(3) The sections are placed in pure pyridine for three hours to be mordanted at 37°C., and washed for one hour in distilled water to remove the odor of the pyridine.

(4) Selected sections are placed in a darkly covered bottle of freshly made and filtered 2 per cent silver nitrate solution, and left for eighteen hours to be impregnated at 37°C.

(5) These are then washed rapidly in distilled water and transferred for from five to fifteen minutes, to the following ammoniacal silver bath: To 5 c.c. of 10 per cent silver nitrate, add 3 drops of fresh 40 per cent sodium hydroxide, in which a precipitate will form. Decant, wash the precipitate several times and add up to 20 c.c. distilled water. Redissolve the precipitate by adding ammonium hydroxide drop by drop, not exceeding 15 drops, and filter through a filter paper previously moistened with distilled water.

(6) The sections are washed rapidly in distilled water and placed in 10 per cent formalin, to reduce for ten minutes, or until the white cloud disappears and the sections turn black.

(7) It is essential to filter all solutions and baths, to use glass needles and to agitate sections constantly after step 4. Wash sections well, usually about fifteen minutes after formalin reduction and differentiate in a 1 per cent solution of cyanide of potassium for from one to four minutes. (This is a very important step.)

(8) The sections are then washed for two minutes in distilled water and placed in a 0.5 per cent solution of gold chloride to which 3 drops of acetic acid have been added, for ten minutes, or until the sections turn gray.

(9) The sections are now washed in water for one minute; and further reduced in 5 per cent sodium-thiosulphate; again washed well in water and counterstained very lightly with hematoxylin.

(10) The sections are next dehydrated quickly in 95 per cent absolute alcohol, cleared in oil of bergamot, and mounted from xylene with Canada balsam.

Nerve fibers are stained characteristically black; other fibers and reticulum, pale brown or darkly yellowish. Where nerve and other fibers are intimately associated, careful focusing will, in properly differentiated slides, permit the distinction between them.

<sup>83</sup> Oertel, H. *Canad. M. A. J.*, 18: 145, 1928.

15. **Transplantation of Tooth-germ Elements.** Homogenous and heterogenous transplantation of tooth-germ elements was carried out by Legros and Magitot<sup>84</sup> in 1874. The odontogenetic tissues were obtained from pups twenty-one to twenty-eight days after birth. In the heterogenous experiments the explants were grafted subcutaneously into guinea pigs or adult dogs. Without the knowledge of modern aseptic procedure it is remarkable that these men obtained positive results in 7 of 26 cases of homogenous grafts of the entire tooth-germ follicle. Tooth development was actually observed in these cases. In 3 of 16 cases whereby pulp tissue was removed and transplanted, dentine was formed by the transplant. Heterogenous grafting proved unsuccessful.

Huggins, McCarroll and Dahlberg<sup>85</sup> followed the autogenous method of grafting different odontogenetic elements in the connective tissue of the abdominal wall of young pups. The formation of dentine and enamel was achieved. For the development of the latter the presence and contact of the ameloblasts with the ameloblastic layer proved to be a *conditio sine qua non*. Otherwise cytomorphosis occurred in the high cylindrical enamel forming cells resulting in epithelial structures resembling the gingiva, ameloblastomata and related structures. The odontoblasts were found capable of survival and function as such. The authors describe their methods as follows:

"Young dogs between the ages of three and six weeks were used and all operations were done under ether anesthesia. An aseptic manner of operating in the mouth was impossible to attain and was found not necessary; the grafts were extraordinarily free from infection. The instruments used in the mouth were sterilized by heat, the operative field was swabbed with iodine and after isolation of the dental follicle, the tissues were removed with fresh instruments into sterile Ringer's solution. The abdominal operation was then done with asepsis.

"The material grafted consisted in the soft tissues of the unerupted permanent canine tooth which was selected because of its large size and ease of accessibility. The canine tooth in the upper jaw was always found anterior and superior to the root of its deciduous precursor; to remove this tooth a 2.5 cm. incision was made in the gingiva covering the lateral aspects of the superior maxilla parallel to the root of the temporary canine tooth, down to the bone; the periosteum was reflected and the thin plate of bone overlying the tooth removed intact. Removal of the tooth in the mandible was slightly more difficult; after making parallel incisions in the gum margin, medial and lateral to the temporary teeth, the periosteum was reflected from the mandible, and a wedge-shaped piece of bone was sawed away just posterior to the deciduous canine, exposing the permanent tooth in the medial aspect of the mandible. Wounds in the jaw were not sutured.

"The dental follicles were opened and the calcified cone of dentine and

<sup>84</sup> Legros, G., and Magitot, E. *Compt. rend. Acad.*, 78: 357, 1874.

<sup>85</sup> Huggins, C. B., McCarroll, H. R., and Dahlberg, A. A. *J. Med.*, 60: 199, 1934.

enamel was always discarded. In a number of cases, an x-ray photograph of the soft tissue was made before transplantation, and in all cases some tissue was excised from the graft for histological control. It was regularly possible to cleanly remove the pulp with its odontoblast layer from the dentine without adherent calcified particles, but it was much more difficult to dissociate calcified enamel from the ameloblast layer, and in many cases a few small bundles of preformed enamel were transplanted inadvertently with the epithelial layer.

"The abdominal site for reception of the transplant was prepared by making a 2 cm. incision in the skin and external oblique muscle. The grafts were inserted between the internal and external oblique muscles, and the latter was then sutured as well as the skin with fine silk.

"The animals were kept from two to fifty-six days on a stock diet of meat scraps, bread and lettuce, with an unlimited supply of cow's milk. A special group in which the known vitamin content of the food was maintained at a high level, was fed in addition by daily gavage a supplementary ration consisting of butter, 12 gm., tomato juice 12 c.c., fresh yeast 4 gm., irradiated ergosterol (1000 D) 2 drops.

"The soft tissues of the developing tooth at this stage consist of (a) the pale white pulp with odontoblasts on the surface and (b) the red epithelial layer consisting of an innermost cylindrical cell layer, the ameloblasts; next a cuboidal row of cells, the stratum intermedium; and outermost a thin layer of flattened epithelial surrounded by connective tissue with a rich vascular supply. The odontoblast-pulp membrane is joined at its base, where the blood vessels and nerves enter, with the more embryonic portion of the epithelial layer, the epithelial sheath of Hertwig, precursor of the tooth root. The membranes are separated normally by the more or less calcified tooth shell of dentine and enamel which in these experiments was always removed and discarded. The relationship of these membranes in the graft was, as will be shown, a determining factor in the product of the transplantation. The membranes were transplanted in various combinations and the distinct difference in color made for the ease in anatomical separation. First, the membranes were cut across so that the epithelial layer (Method A) and the pulp-odontoblast layer (Method B) could be transplanted to different parts of the abdominal wall. Secondly, the membranes were transplanted together (Method C) so that the epithelial layer had a quasi-normal relationship to the pulp and the cylindrical ameloblasts were in close proximity to the odontoblasts; and in another series the epithelial layer instead of ensheathing the pulp was drawn upwards so that these membranes were in continuity but not approximated (Method D). Thus in the experiments done using Method D the epithelial layer bore a 'skin-the-rabbit' relationship to the pulp. In another series (Method E), a transverse section of the pulp was circumsised so that all of the odontoblasts were removed together with a thin layer of underlying pulp cells; thus the surface cells of the pulp were transplanted to a different location from that of the central pulp cells. In Method F, a sheet of mouth epithelium covering the mandible was excised and transplanted to the abdominal wall."

In 1933 Nakamura<sup>86</sup> rendered an account of his experiments on "auto-plastic" transplantation of the persistent mandibular incisor tooth-germ of the rabbit to the brain, spleen and liver. He used 93 Manchurian rabbits. Enamel, dentine, osteodentine, the sheath of Hertwig, stellate reticulum, stratum intermedium, dentogingival lamina, squamous epithelium, ameloblastomatous structures, epithelial rests, dental pulp tissue and osteoid tissues developed. Cementum was never encountered. No appreciable absorption was noticed up to the eighty-third day. The formation of hard structures was greater in the spleen than in the brain or liver.

In 1934 Nakamura<sup>87</sup> reported on "auto- and homoioplastic" transplantation experiments for which 81 rabbits were used. Observations were made over one to 104 days, after which absorption and necrosis were noticed.

**16. Development of Tooth Germs in Vitro.** The only report on this subject is that of Glasstone.<sup>88</sup> His material and methods are quoted herewith:

"The tooth germ of eighteen to twenty-one day rat embryos were cultivated. Molars and incisors which had not yet begun to form dentine were explanted whole; in those incisors where a cap of dentine had formed on the tip, this cap together with the enamel epithelium was removed and the dentine papilla itself was explanted. In performing this last operation some of the odontoblasts remained adherent to the cap of dentine whilst some were left behind on the tip of the dentine papilla. In some of the explants a little of the enamel epithelium also remained attached. Owing to the small size of the dentine papilla it was not possible to remove the ameloblast completely without irreparable damage to the tissue.

"As extirpation had to be done with the strictest aseptic precautions and the removal of teeth from post-fetal rats under such conditions is almost impossible, the necessary material had to be obtained from embryos.

"The tooth germs for cultivation were dissected out with a cataract knife and a needle from both upper and lower jaws and washed in saline. In order to determine the stage of development of each tooth one tooth was removed for extirpation, whilst the corresponding tooth from the opposite side of the jaw was fixed either in situ in a fragment of the jaw or after removal and sectioned to serve as control.

"The culture methods employed were the hanging-drop and watch-glass techniques. In the hanging-drop method the explants were placed in a clot of medium composed of 1 drop of fowl plasma and 1 drop of embryo extract made with Pannett and Compton's saline from ten to eleven day chick embryos. The cultures were grown on 1¼ inch square cover slips inverted over a hollow

<sup>86</sup> Nakamura, G. *Kokubyo-Gakkai-Zasshi*, 7: 12, 1933.

<sup>87</sup> *Kokubyo-Gakkai-Zasshi*, 8: 94, 1934.

<sup>88</sup> Glasstone, S. J. *Anat.*, 70: (pt. 2) 260, 1936.



ground slide sealed with paraffin wax and incubated at 37°C. The explants were transferred to fresh medium every seventy-two hours.

"In the watch-glass method 4 drops of fowl plasma and 4 drops of embryo extract were mixed in a watch-glass which was enclosed in a Petri dish surrounded with cotton-wool saturated in sterile water. A large hole was cut in the cotton-wool to allow transillumination of the explants for microscopical observation. The tissue which was grown on the surface of the clot was removed from the old medium every seventy-two hours, washed in saline and transferred to a fresh clot.

"The explants were fixed at different periods of growth in Zenker's solution containing 3 per cent glacial acetic acid for thirty minutes, then washed, dehydrated, cleared in cedar-wood oil and imbedded in paraffin wax. Serial sections were cut and were stained with iron hematoxylin and chromotrope."

Whole or partial tooth germs cultivated showed remarkable powers of histological differentiation.

**17. Precipitin Reactions with Bone and Teeth.** Plathner<sup>89</sup> used freshly extracted teeth which were thoroughly cleaned of bone, soft tissue and blood and then pulverized until finally the powder passed through a silk screen of 0.15 mm. mesh. To this powder was added a physiological salt solution in a 2:3 ratio. Both were then agitated for twenty-four hours to increase the solubility of the powder. After centrifuging under sterile precautions the extract was removed into a sterile tube and again centrifuged. The albuminous content of the extract was then determined by the Hauscher capillary method. Antisera were used with a titer of 1:20,000.

Fresh human and animal teeth gave within twenty-four hours a species specific precipitin reaction. Intact human molars formed exceptions, also enamel. The author believes that teeth long buried in the ground or exposed to the air may not give any reactions.

Henrici<sup>90</sup> tested the feasibility of determining the origin of archeologic relics composed of bone or ivory by means of immunological reactions.

A fox's skull was thoroughly cleaned of all adherent soft tissue, the bone crushed and ground to powder (the largest particles of powder were  $\pm$  0.5 mm. in diameter). The teeth were ground separately. Bone and teeth were then extracted with a volume of salt solution slightly less than the volume of ground material, for two hours at room temperature, shaken repeatedly, and then kept over night in the ice box. Precipitin tests with these extracts were completely negative with the teeth, and very faintly positive with the bone, the reaction with the latter being about the same in degree as with fox-serum diluted 1:100,000.

<sup>89</sup> Plathner, H. *Deutsch. Ztschr. f. d. ges. gerichtl. Med.*, 23: 61, 1934.

<sup>90</sup> Henrici, A. T. *Proc. Soc. Exper. Biol. & Med.*, 34: 343, 1936.

The precipitate was obtained by injecting four rabbits with fox-serum twice weekly for twelve weeks. The pooled serums precipitated with fox-serum in dilutions up to 1:500,000.

The experiment indicated that the precipitin test, while not absolutely hopeless, is not very promising.

**18. Capillaroscopy.** With regard to changes in the parodontia of teeth, retarded development of the jaws, oral symptoms of metallic poisoning and to investigate the clinical value of capillaroscopy, capillaries of the gingiva and lips have been studied both in vivo and in sectioned material.<sup>91</sup>

<sup>91</sup> Schulze, J. W. Über die Beobachtung der Kapillaren an Zahnfleisch des lebenden Menschen. *Zhnärztl. Rndsch.*, 34: 803-6, 1925.

Bartoschek, T. Kapillaroskopie als klinische Untersuchungsmethode. *Przeglad Dentystyczny*, No. 10, 1929. Abstr. *Zhnärztl. Rndsch.*, 39: 394-5, 1930.

Bettmann. Capillarmikroskopische Befunde an der Lippenschleimhaut. *Ztschr. f. Anat. u. Entwickl.*, 91: 391-40, 1929. Abstr. *Deutsche Monatschr. f. Zahnh.* 48: 1244, 1930.

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Scholl, H. Über das Verhalten der Zahnfleischkapillaren bei Sulfidsaumbildung. *Paradentium* (Sonderbeil. d. Zahnärztl. Rdsch.), 2: 86-91, 1930. Abstr. *Deutsche Monatschr. f. Zahnh.*, 48: 1523, 1930.

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# METHODS FOR THE INTERCELLULAR SUBSTANCES OF THE CONNECTIVE TISSUES

F. B. MALLORY AND FREDERIC PARKER, JR.

Fibroglia fibrils 402. Collagen 404. Elastic fibrils 413. Mucin 416. Amyloid 417.

It is probable that all the intercellular substances of the connective tissues are formed by one protean cell, the fibroblast. It produces fibroglia, collagen and elastic fibrils, mucus, the ground substances of cartilage and bone and, under certain pathologic conditions, amyloid. Reticulum is a term applied to that form or arrangement of collagen which is colored intensely by silver stains apparently simply as the result of a physical condition, namely, separation into single fibrils or minute strands of them by cells, elastic fibrils or fluid.

## I. Fibroglia Fibrils

Fibroglia fibrils are in intimate relation with the cytoplasm of the fibroblast, running over its surface and extending out along its processes in the same way that neuroglia fibrils do. They are very delicate, straight or slightly curved, run in parallel lines and are definitely separated from one another. They seem to pass from cell to cell, thus aiding to form a syncytium.

Fibroglia fibrils are most evident when fibroblasts are leading an active existence as in granulation tissue, in the organization of fibrin (thrombi, etc.) in the stroma of cancers and in fibrosarcomas if the latter are not growing too rapidly. In the spleen and kidneys they can readily be demonstrated, but in certain tissues such as the heart, the endometrium, the ovary, etc. they are not in evidence under normal conditions.

In order to demonstrate fibroglia fibrils the tissue must be absolutely fresh for best results, that is, removed surgically and placed in a fixative within one to five minutes after it is taken from the living body. The reason for this is that the fibrils quickly lose their staining properties post mortem.

1. **Fixation.** The only fixative recommended is Zenker's fluid and the tissue must be cut into thin sections not over 2 to 4 mm. thick. Fair results can be obtained by Zenkerizing formaldehyde fixed tissue but the procedure is not to be encouraged.

- (1) Zenker's fluid, twenty-four hours.
- (2) Running water, twenty-four hours.
- (3) Alcohol, 80 per cent, twenty-four hours.

**2. Staining.** There are two staining methods which are particularly useful and more or less specific for the demonstration of fibroglia fibrils: a, phosphotungstic acid hematoxylin, which led to their discovery, and b, acid fuchsin followed by permanganate of potassium. Two other stains often bring them out with great distinctness, namely, the eosin (phloxin)-methylene blue method which colors them a bright pink, and the aniline blue collagen stain by which method they are dyed an intense red by the acid fuchsin.

*a. Mallory's Phosphotungstic Acid Hematoxylin Stain.*

Water .....	100.0 c.c.
Hematoxylin .....	0.1 gm.
Phosphotungstic acid crystals .....	2.0 gm.

Dissolve the hematoxylin by the aid of heat in part of the water and add it after it is cool to the acid dissolved in the rest of the water. No preservative is necessary. The solution requires several weeks in order to ripen, but may be ripened at once by the addition of 10 c.c. of a 0.25 per cent aqueous solution of permanganate of potassium.

Hematein ammonium may be used instead of hematoxylin but requires 5 c.c. of the permanganate solution to ripen it fully at once.

*Staining Method.* Zenker fixation, paraffin sections. Treat sections with iodine in the usual way in order to remove the mercury precipitate and then extract the iodine by means of alcohol or a 0.5 per cent solution of hyposulphite of sodium and finally get them into water.

- (1) Place sections in a 0.25 per cent aqueous solution of permanganate of potassium for five to ten minutes.
- (2) Wash in water.
- (3) Oxalic acid, 5 per cent aqueous solution, ten to twenty minutes.
- (4) Wash thoroughly in several changes of water.
- (5) Stain in phosphotungstic acid hematoxylin for twelve to twenty-four hours.
- (6) Transfer sections directly to 95 per cent alcohol, followed by absolute. Dehydrate quickly because alcohol readily extracts the red part of the stain.
- (7) Clear in xylol and mount in xylol balsam.

The solution stains nuclei, centrosomes, achromatic spindles and fibroglia, myoglia and neuroglia fibrils, fibrin, and the contractile elements of striated muscle blue, collagen (including reticulum), and the ground substances of cartilage and bone varying shades of yellowish to brownish red. Coarse elastic fibrils are sometimes colored a purplish tint.

If celloidin sections are used, clear with xylol from 95 per cent alcohol by means of the filter paper blotting method. Origanum and other oils cause the blue stain to fade.

Fair results may sometimes be obtained after formaldehyde fixation if the tissues are first carried through Zenker's fluid but the method is not recommended.

*b. Mallory's Acid Fuchsin Stain.* Zenker fixation, paraffin sections.

(1) Stain in a 1 per cent aqueous solution of acid fuchsin overnight in the cold or for one hour in the paraffin oven (54°C.).

(2) Drain slides and differentiate in a 0.1 per cent aqueous solution of permanganate of potassium for forty to sixty seconds. This step must not be prolonged beyond the exact time needed or the section will be decolorized.

(3) Dehydrate in 95 per cent alcohol followed by absolute.

(4) Clear in xylol and mount in xylol balsam.

Nuclei and fibroglia fibrils red, collagen pale reddish yellow, elastic fibrils bright lemon yellow, red blood corpuscles purplish red.

## II. Collagen

Collagen consists of exceedingly delicate fibrils which usually present a wavy appearance. They may be more or less widely separated from each other, or united into thin or coarse strands, or fused into fibrillar or homogeneous masses. Reticulum is the term applied to collagen which has been stained by the silver method and this occurs only under certain physical conditions, namely, when the individual fibrils or small strands of them are separated from each other by cells, elastic fibrils or fluid, but not when they are compacted. The appearance of the deeply stained fibrils is so striking in contrast with the lightly staining denser masses of collagen that they have very generally been assumed to be of a different chemical nature without taking into consideration the results obtainable by the application of other staining methods for collagen. Their origin has been attributed by many to endothelial cells or to reticulum cells.

1. **Fixation.** Collagen fibrils retain their characteristic staining properties a long time post mortem but absolutely fresh tissue should be preferred for the study of them whenever obtainable. Any fixative may be employed, but Zenker's fluid is recommended over all others. A variety of more or less specific stains is available for demonstrating them. The oldest and most generally useful is Van Gieson's because it works well after all the ordinary fixatives, but it does not stain the fibrils so intensely as is desirable.

2. **Staining.** *a. Van Gieson's Picro Acid Fuchsin Solution.* This was originally made by adding to a saturated aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet-red color. Nowadays it is usual to employ a 1 per cent aqueous solution of acid fuchsin and to add 5 to 15 c.c. of it to 100 c.c. of a saturated aqueous solution of picric acid according to the intensity of stain desired, or the nature of the fixative employed.

Staining method:

- (1) Stain paraffin sections rather deeply with alum hematoxylin.
  - (2) Wash in water.
  - (3) Stain in Van Gieson's solution for five minutes or more.
  - (4) Transfer directly to 95 per cent alcohol followed by absolute.
  - (5) Clear in xylol and mount in xylol balsam.
- For celloidin sections clear in origanum oil after 95 per cent alcohol.

The two drawbacks to this method are that the red stain of the collagen is not intense enough and that it tends to fade. This latter fault may be obviated in the future by employing the new acid fuchsin, Lot #39, recently introduced by French and guaranteed not to fade.

*b. Mallory's Aniline Blue Collagen Stain.* Zenker fixation.

(1) Stain paraffin or celloidin sections in a .25 per cent aqueous solution of acid fuchsin for thirty minutes.

(2) Drain and pass sections directly to the following solution:

Water .....	100	c.c.
Aniline blue W.S. ....	0.5	gm.
Orange G (85-90 per cent dye content) .....	2	gm.
Phosphotungstic acid (Merck) .....	1	gm.

Stain for one to twenty-four hours, or longer if the deepest color obtainable is desired. An hour in the paraffin oven gives as good a result as overnight in the cold.

(3) Transfer directly to 95 per cent alcohol, two or three changes, until no more color is given off.

(4) Absolute alcohol, xylol, neutral Canada balsam.

For celloidin sections use 95 per cent alcohol and clear in oleum origani cretici or by the blotting paper xylol method.

Collagen fibrils, the ground substances of cartilage and bone, mucin, amyloid and certain other hyaline substances are stained varying shades of blue; nuclei, fibroglia, myoglia and neuroglia fibrils, axis cylinders and fibrin red; red blood corpuscles and myelin yellow; elastic fibrils pale pink or yellow. If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid fuchsin.

This staining method as used for many years contained phosphomo-

lybdc acid which often caused fading of the acid fuchsin. Phosphomolybdic acid has been substituted for it with beneficial results.

c. *Verocay's<sup>1</sup> Stain for Collagen*. Any fixative can be used; and frozen, celloidin or paraffin sections. All sections must be closely attached to the slip before staining in order to prevent shrinking by the mordant, paraffin sections by means of egg albumen in the usual way, and then in addition, like the frozen and celloidin sections, by means of a thin solution of celloidin poured over them after they have been pressed onto the surface of clean, fat-free slips. Harden the celloidin in chloroform followed by 80 per cent alcohol.

- (1) Wash sections thoroughly in water.
- (2) Mordant them in a 1 per cent aqueous solution of chromic acid at 46°C. or thereabouts for ten to twenty-four hours or more.
- (3) Wash thoroughly in several changes of water.
- (4) Stain in Delafield's hematoxylin (which must not be too old) for one-half to one hour or longer.
- (5) Dehydrate in 95 per cent alcohol. Clear by the blotting paper xylol method and mount in xylol balsam.

Collagen is stained dark blue by this method.

d. *Mallory's Phosphomolybdic Acid Hematoxylin Stain for Collagen*. Zenker fixation, paraffin sections.

- (1) Stain sections in the following solution for twelve to twenty-four hours or more, or in the paraffin oven at 54°C. or thereabouts for two to three hours.

Hematoxylin .....	1.0 gm.
Phosphomolybdic acid crystals .....	2.0 gm.
Water .....	100 c.c.

The solution requires several weeks in order to ripen but may be ripened at once by the addition of 5 c.c. of a 1 per cent solution of permanganate of potassium.

- (2) Wash in water.
- (3) Decolorize and dehydrate in 95 per cent alcohol followed by absolute.
- (4) Clear in xylol and mount in xylol balsam.

It is much simpler than Verocay's method and is believed to yield equally good results. Single collagen fibrils are brought out sharply, a deep blue color, but not so well as by the silver method. If a counter-stain is desired, place sections first in a 0.1 per cent aqueous solution of acid fuchsin for five to ten minutes and then drain and transfer directly to the hematoxylin solution.

e. *Silver Impregnation Methods for Collagen (Reticulum)*. Bielschow-

<sup>1</sup> Verocay. *Centralbl. f. allg. Path.*, p. 942, 1906.

sky<sup>2</sup> originally devised his silver impregnation methods for the study of neuro-fibrils, but Maresch<sup>3</sup> applied these methods to connective tissue for the study of the finer fibrils of collagen, the so-called reticulum. Reticulum fibers are brought out by these methods more clearly and beautifully than by any other and study of such preparations is a revelation as to the complexity and number of these fibers. Bielschowsky's method depends on the reduction by formalin of the easily reducible soluble silver salt formed by dissolving with ammonia the precipitate caused by adding a solution of sodium hydroxide to a solution of silver nitrate. Numerous modifications of his methods have been devised but the basic principles remain the same.

Certain precautions must be taken in carrying out these methods, for, as is well known, silver is easily affected by organic matter. Some of the main points to be observed are given below:

(1) All glassware must be chemically clean. This is best accomplished by treating it with sulphuric-acid-potassium-bichromate cleaning solution, followed by thorough washing with tap water, then distilled water.

(2) The distilled water must be free from organic matter. This can be tested by adding a few drops of a silver nitrate solution and watching for a color change or precipitate. Da Fano recommends using water re-distilled on potassium permanganate.

(3) All chemicals must be of the highest chemical purity.

(4) In handling frozen or celloidin sections, glass instruments, never metal, should be used.

(5) Certain filter papers contain sufficient organic matter to affect the silver solutions and such should be avoided.

(6) Filter all solutions.

(7) In our experience equally good results can be obtained by using either yellow or brown chloride of gold.

Of the numerous modifications of Bielschowsky's methods, we are giving but five below. These five would seem to be sufficient for ordinary laboratory work. If further methods are desired, the reader should consult more special works. For laboratories where Zenker fixed tissue and paraffin sections are the rule, we can recommend highly Foot's modification given later (p. 410).

(1) Bielschowsky-Maresch Method. Fixation in 10 per cent formalin is the best. Maresch states that alcohol fixed tissue may also be used but sections from such tissue should be put in 10 per cent formalin for several hours followed by washing in distilled water before being stained. Material fixed in sublimate, osmic acid or chrome salts is not satisfac-

<sup>2</sup> Bielschowsky, M. J. *f. Physiol. u. Neurol.*, 3: 169, 1904.

<sup>3</sup> Maresch, R. *Centralbl. f. allg. Pathol. u. path. Anat.*, 16: 641, 1905.



tory, as impregnation is uneven and the silver precipitates out in fine discrete granules.

Frozen sections give the best results but celloidin sections can be used and it is not necessary to remove the celloidin. Paraffin sections may also be used.

Bielschowsky's ammoniacal silver bath used in step (5) is made as follows:

To 10 c.c. of a 10 per cent solution of silver nitrate add first 5 drops of a 40 per cent aqueous solution of sodium hydroxide, then strong ammonia drop by drop, stirring constantly with a glass rod, until the precipitate is just dissolved; an excess of ammonia is to be avoided and this is best done by stopping the addition of the ammonia while there is a granule or two of the precipitate still undissolved. Dilute to 25 c.c. with distilled water and filter. This solution must be made fresh each time before use.

The reduction in step (7) is carried out in a neutral solution of formalin, as the free acid which often occurs in commercial formol interferes with the reducing process.

The gold chloride bath is made up of 10 c.c. of distilled water to which is added 2 drops of a 1 per cent solution of gold chloride and 2 to 3 drops of acetic acid.

The complete procedure follows:

- (1) Wash formalin fixed tissue several hours in running water before freezing.
- (2) Cut frozen sections, receiving the sections in distilled water.
- (3) Place sections for twenty-four hours in a 2 per cent solution of silver nitrate in the dark.
- (4) Rinse quickly in distilled water.
- (5) Transfer sections to ammoniacal silver bath for two to thirty minutes depending on their thickness. Here they become yellowish brown.
- (6) Rinse quickly with distilled water.
- (7) Reduce in a 20 per cent solution of formalin in tap water for a few minutes up to half an hour, until no more white clouds appear. The preceding rinse in distilled water in step (6) acts as a kind of differentiation for this reducing process. If the rinsing in step (6) is too short, the sections will be too dark; if too long, the sections will not be dark enough; as a general rule, the time should be short.
- (8) Wash in distilled water.
- (9) Tone in the acid gold bath until the background becomes red-violet; with sections 10 $\mu$  thick, ten minutes usually suffices.
- (10) Wash in distilled water.
- (11) Place for fifteen to thirty seconds in a 5 per cent solution of sodium hyposulphite.

- (12) Wash thoroughly in tap water.
- (13) Dehydrate, clear and mount as usual.

By this method, the connective tissue fibers, even the finest, are a deep black on a clear background; thick collagen bundles are more chocolate brown or violet. Nuclei as a rule are not stained to any degree and aniline dyes can be used for staining these.

(2) Perdrau's<sup>4</sup> Modification of Bielschowsky's Method (as given by Bailey and Hiller).<sup>5</sup>

Material should be fixed in 10 per cent neutral formalin.

The following special reagents will be needed:

(1) Pal's decolorizer—equal parts of a 1 per cent aqueous solution of oxalic acid and a 1 per cent aqueous solution of acid potassium sulphite.

(2) Bielschowsky's ammoniacal silver bath, made as follows: To 5 c.c. of a 20 per cent solution of silver nitrate add first, 2 drops of a 40 per cent solution of sodium hydroxide and then strong ammonia drop by drop until the precipitate is just dissolved; dilute to 50 c.c. with distilled water and filter. This reagent must be prepared each time before use.

The procedure is as follows:

(1) Wash blocks of formalin fixed tissue twelve to twenty-four hours in running tap water, then twenty-four hours in distilled water, changing the water several times.

(2) Cut frozen sections  $15\mu$  to  $25\mu$  in thickness.

(3) Wash in distilled water twenty-four hours.

(4) Treat sections ten minutes with a 0.25 per cent solution of potassium permanganate.

(5) Wash in distilled water.

(6) Place in Pal's decolorizer until white.

(7) Wash thoroughly overnight in several changes of distilled water.

(8) Place in a 2 per cent solution of silver nitrate in the dark for twenty-four hours.

(9) Wash in distilled water not more than five minutes.

(10) Treat sections forty to sixty minutes with Bielschowsky's ammoniacal silver solution.

(11) Wash quickly in distilled water.

(12) Reduce thirty minutes in a 20 per cent solution of formalin (not neutral) made with tap water.

(13) Wash in distilled water.

(14) Tone in a 1:500 solution of gold chloride until sections are an even violet color.

(15) Wash in distilled water.

<sup>4</sup> Perdrau, J. R. *J. Path. & Bacteriol.*, 24: 117, 1921.

<sup>5</sup> Bailey, P., and Hiller, G. *J. Nerv. & Ment. Dis.*, 59: 337, 1924.

- (16) Fix in a 5 per cent solution of sodium hyposulphite.
- (17) Wash thoroughly in distilled water.
- (18) Dehydrate, clear, and mount as usual.

By this method, the broader bands of collagen are stained reddish and the finer strands, so-called reticulum, black. This method may also be used on paraffin sections if the slides are not agitated in the solutions, otherwise the sections will come off the slide.

(3) Foot's Modification of Bielschowsky's Method. This method was devised especially for Zenker fixed tissue. Formalin fixed tissue may also be used and in our hands has proved quite satisfactory. This method is intended for paraffin sections; we have had no experience with its use on frozen or celloidin sections.

The ammoniacal silver bath used is prepared as follows: To 20 c.c. of a 10 per cent solution of silver nitrate add 20 drops of a 40 per cent solution of sodium hydroxide. The resulting brownish precipitate is dissolved in strong ammonia, which is added slowly, shaking continually; about 2 c.c. will be needed so it is well to add the ammonia drop by drop as this point is neared until the precipitate is almost dissolved. It is better to filter out a few undissolved grains than to run the risk of adding too much ammonia. The resulting solution is made up to 80 c.c. with distilled water and filtered before use. This solution must be made fresh each day as it does not keep.

The solution of formalin used for reduction should be neutral and it is well to change it after the first ten or fifteen minutes.

- (1) Remove paraffin from the sections in the usual manner.
- (2) Weak alcoholic iodine solution for five minutes.
- (3) Weak aqueous solution of sodium hyposulphite until sections are white.
- (4) Wash in tap water.
- (5) Treat sections with a 0.25 per cent solution of potassium permanganate for five minutes.
- (6) Rinse in tap water.
- (7) Place sections in a 5 per cent solution of oxalic acid for fifteen to thirty minutes.
- (8) Wash thoroughly in tap water.
- (9) Rinse in distilled water. Use distilled water for washing until after sections have been treated with formalin.
- (10) Leave sections for forty-eight hours in a 2 per cent solution of silver nitrate (in subdued light, but not in the dark).
- (11) Wash a short time in distilled water.
- (12) Ammoniacal silver solution for thirty minutes.
- (13) Wash quickly in distilled water.
- (14) Reduce in a 5 per cent neutral formalin solution for thirty minutes.
- (15) Rinse at tap.

- (16) Tone in a 1 per cent solution of gold chloride for one hour.
- (17) Rinse at tap.
- (18) Remove excess silver by treating sections with a 5 per cent solution of sodium hyposulphite for two minutes.
- (19) Wash thoroughly several hours in running tap water.
- (20) Stain in Harris' hematoxylin ten minutes, or Weigert's iron hematoxylin one minute.
- (21) Soak in tap water until blue.
- (22) Counterstain in Van Gieson's picro-acid fuchsin solution for thirty seconds.
- (23) Dehydrate, clear and mount as usual.

Coarser collagen stains red to rose, finer collagen, so-called reticulum, black to dark violet; nuclei black, blue or brownish; cytoplasm, grayish yellow; muscle fibers and elastic fibers, more brightly yellow. The finest reticulum fibers are brought out by this method, for if the silver does not penetrate them, the acid fuchsin will, so they are sometimes beaded black on vermillion.

Instead of staining with hematoxylin and Van Gieson's solution, Mallory's acid fuchsin-aniline blue collagen method can be used, especially for the study of fibroglia and myoglia fibrils and for the relation of these to collagen. Sometimes, especially for photographic purposes, it is of advantage to omit the counterstaining with Van Gieson's solution.

(4) Foot's<sup>6</sup> Modification of the Hortege Silver Carbonate Method. This method has the advantage over the preceding one because of its saving in time. It can be carried out in less than an hour while the other method requires three days. The results are as satisfactory, if not more so.

The method was devised especially for paraffin sections of Zenker-fixed material.

The silver-ammonium carbonate which must be prepared fresh for each batch of slides is made as follows:

To 10 c.c. of a 10 per cent aqueous solution of silver nitrate, add 10 c.c. of a saturated aqueous solution of lithium carbonate. The resulting heavy white precipitate is allowed to settle and the supernatant fluid is poured off. The precipitate is then washed several times with 25 to 50 c.c. of distilled water, the precipitate being allowed to settle each time and the supernatant fluid poured off. After 25 c.c. of fresh distilled water is added, this washed precipitate is almost dissolved in strong ammonia water, which is added drop by drop while the container is shaken vigorously. About 8 to 15 drops of the ammonia will be needed and great care must be taken not to overstep the end-point of solution. It is better to leave a few grains undissolved than to add too much ammonia. The

<sup>6</sup> Foot, N. C. *J. Lab. & Clin. Med.*, 9: 777, 1923-1924.

Foot, N. C., and Ménard, B. S. *Arch. Path. & Lab. Med.*, 4: 211, 1927.

entire solution is then made up to 100 c.c. with distilled water and heated to 50°C. The slides are immersed in this solution and placed in an incubator at 37.5°C., from ten to fifteen minutes, until they turn a yellowish gray. The temperature of the bath will remain between 40° and 50°C., under these circumstances.

The procedure is as follows:

- (1) Remove paraffin from sections in the usual manner.
- (2) Weak alcoholic iodine solution for five minutes.
- (3) Bleach with 5 per cent aqueous sodium hyposulphite.
- (4) Wash in tap water.
- (5) Treat with 0.25 per cent aqueous potassium permanganate solution for five minutes.
- (6) Rinse with tap water.
- (7) Leave sections in 5 per cent aqueous oxalic acid solution for ten minutes.
- (8) Wash well at tap followed with a wash in distilled water.
- (9) Treat sections for ten to fifteen minutes with silver-ammonium carbonate at about 45°C.
- (10) Rinse quickly in distilled water.
- (11) Reduce in 20 per cent neutral formalin for two minutes.
- (12) Wash well with tap water.
- (13) Tone in 1:500 aqueous gold chloride solution for two minutes.
- (14) Wash in tap water.
- (15) Fix in 5 per cent aqueous sodium hyposulphite for two minutes.
- (16) Wash well in tap water
- (17) Counterstain, dehydrate and mount as in preceding method.

The staining of the collagen and other elements is the same as in the preceding method.

(5) Wilder's Silver Stain for Reticulum Fibers.<sup>7</sup> Fixation: Fix tissues in 10 per cent formalin, acetic-Zenker or formol-Zenker.

Imbedding Tissues, Cutting and Mounting Sections: Imbed in paraffin or celloidin, or cut frozen sections. Paraffin sections are mounted according to the routine method with Mayer's glycerin-albumin and are run through xylol, graded alcohols and distilled water before staining. Celloidin sections may vary in thickness from 4 to 30 microns. The thick sections give a better idea of the density of the fibers in some tumors. They are stained in dishes before mounting. Frozen sections may be stained in dishes before mounting, or mounted on slides and attached with thin celloidin before staining.

Pretreatment: Place the sections in 0.25 per cent potassium permanganate or in 10 per cent phosphomolybdic acid for one minute. Rinse in distilled water and place in hydrobromic acid (Merck's concen-

<sup>7</sup> Wilder, H. C. *Am. J. Path.*, 11: 817, 1935.

trated, 34 per cent, 1 part; distilled water, 3 parts) for 1 minute. Hydrobromic acid may be omitted following the use of phosphomolybdic acid.

Sensitization: Wash in tap water, then in distilled water and dip in 1 per cent uranium nitrate (sodium free) for five seconds or less.

Impregnation: Wash in distilled water ten to twenty seconds and place in silver diamino hydroxid (Foot<sup>8</sup>) for one minute.

To 5 c.c. of 10.2 per cent silver nitrate add ammonium hydroxide drop by drop until the precipitate which forms is dissolved. Add 5 c.c. of 3.1 per cent sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make the solution up to 50 c.c. with distilled water.

Reduction: Dip quickly in 95 per cent alcohol and reduce for one minute in the following solution:

Distilled water, 50 c.c.; 40 per cent neutral formalin (neutralized with magnesium carbonate), 0.5 c.c.; 1 per cent uranium nitrate, 1.5 c.c.

Toning: Wash in distilled water and place in 1:500 gold chloride (Merck's reagent) for one minute. Rinse in distilled water. Place in 5 per cent sodium thiosulphate (hyposulphite) for one to two minutes.

Counterstaining and Mounting: Wash in tap water; counterstain, if desired, with alum hematoxylin and Van Gieson, or alum hematoxylin and eosin; dehydrate in alcohol. Clear in xylol and mount in balsam. The use of ammonia must be avoided in blueing sections after hematoxylin as it dissolves the silver.

The use of distilled water and clean glassware for all solutions is essential. All the solutions may be used repeatedly and kept in Coplin jars for several days. The solutions keep without disintegrating in amber glass-stoppered bottles for an indefinite time.

### III. Elastic Fibrils

Three excellent methods are available for staining elastic fibrils. Weigert's is the simplest and most generally useful once the staining solution has been made up. Verhoeff's has the great advantage of staining well after Zenker fixation. Unna's is the oldest but has been largely superseded by the other two.

#### 1. Weigert's<sup>9</sup> Method for Elastic Fibrils.

(1) Stain paraffin sections twenty minutes to one hour in a fuchsin-resorcin solution prepared as follows:

<sup>8</sup> Foot, N. C. *Am. J. Path.*, 5: 223-238, 1929.

<sup>9</sup> Weigert, C. *Centralbl. f. allg. Path.*, 9: 287, 1898.

Fuchsin (basic) .....	2 gm.
Resorcin .....	4 gm.
Water .....	200 c.c.

Boil the solution in a porcelain dish; when it is briskly boiling add 25 c.c. of liquor ferri chloridi (a 29 per cent solution); stir and boil for two to five minutes. A precipitate forms. Cool and filter. The filtrate is thrown away. The precipitate remains on the filter-paper until all the water has drained away or until the precipitate has thoroughly dried. Then return filter and precipitate to the porcelain dish, which should be dry, but which should contain whatever part of the precipitate remained sticking to it. Add 200 c.c. of 95 per cent alcohol, and boil. Stir constantly, and fish out the filter-paper as the precipitate is dissolved off. Cool; filter; add alcohol to make up to 200 c.c. Add 4 c.c. of hydrochloric acid.

(2) Wash in several changes of 95 per cent alcohol followed by absolute.

(3) Clear in xylol and mount in xylol balsam.

For celloidin sections clear from 95 per cent alcohol by the blotting paper xylol method.

## 2. Hart's<sup>10</sup> Modification of Weigert's Elastic Tissue Stain.

(1) Stain sections in lithium carmine thirty minutes.

(2) Then directly into

Acid alcohol..... 100 c.c.

Weigert's stain..... 5 c.c.

Stain overnight, twelve hours at least.

(3) Wash in 85 per cent alcohol, then dehydrate, clear and mount as in Weigert's method.

**3. Verhoeff's<sup>11</sup> Elastic Tissue Method.** Fixation in formalin or Zenker's fluid is preferred. Tissues or sections should not be treated with iodine solution before staining. Mercurial precipitates, if removable, are removed by the staining solution. For the best results the solution should be used within twenty-four hours, but satisfactory specimens may be obtained with solutions one month old.

The staining fluid is made as follows:

Hematoxylin crystals..... 1 gm.

Absolute alcohol..... 20 c.c.

Dissolve in test tube by aid of heat, filter, and add in order given:

Aqueous solution (10 per cent) of ferric chloride..... 8 c.c.

Lugol's solution (iodine, 2; potassium iodide, 4; water, 100). 8 c.c.

(1) Immerse paraffin sections in the staining fluid for fifteen minutes or longer until perfectly black.

(2) Wash in water.

(3) Differentiate in a 2 per cent aqueous solution of ferric chloride. This proc-

<sup>10</sup> Hart, *Centralbl. f. allg. Path.*, vol. 19: 1901.

<sup>11</sup> Verhoeff, F. H. *J. Am. Med. Ass.*, 50: 876, 1908.

ess requires only a few seconds. To observe the stages in the differentiation, the sections may be examined in water under a low magnification. If the differentiation has been carried too far, the sections may be restrained, provided that they have not been treated with alcohol.

- (4) Wash in water.
- (5) Place in 95 per cent alcohol to remove iodine.
- (6) Transfer to water for five minutes or longer.
- (7) Counterstain, if desired, in a 0.5 per cent aqueous solution of eosin.
- (8) Dehydrate in 95 per cent alcohol followed by absolute.
- (9) Xylol, xylol balsam.

By this method elastic tissue is stained black, while connective tissue, fibroglia, myoglia and neuroglia fibrils, myelin and fibrin take the eosin stain. Nuclear staining may be obviated by doubling the amount of Lugol's solution in the staining fluid. Degenerated elastic tissue (elacin) is also stained by this method. The degenerated fibrils may be distinguished from the normal by staining less intensely and presenting less distinct outlines.

Equally good results, especially after Zenker's fixation, may be obtained by staining the tissues en masse. Myelin, however, is also stained. Thin slices of tissue after fixation are removed from 80 per cent alcohol and immersed in the staining fluid four days. They are then quickly rinsed in water to remove excess of stain, placed in 80 per cent alcohol and imbedded in the usual manner. The sections are differentiated in a 0.5 per cent solution of ferric chloride.

**4. Unna's<sup>12</sup> Orcein Method for Elastic Fibers.** Unna's method of using orcein is as follows, and can be highly recommended:

- (1) Stain sections in the following solution:

Orcein.....	1 gm.
Hydrochloric acid.....	1 c.c.
Absolute alcohol.....	100 c.c.

Place the sections in a dish and pour over them enough of the solution to cover them. Warm gently in an incubator or over a small flame for ten to fifteen minutes until the solution thickens, or leave in the solution at room temperature overnight.

- (2) Wash off thoroughly in dilute alcohol (70 per cent).
- (3) Wash in water to get rid of all the acid and to fix the color.
- (4) Alcohol.
- (5) Oil.
- (6) Xylol balsam.

The washing in water is not absolutely essential.

<sup>12</sup> Unna, P. G. *Monatschr. f. prakt. Dermat.*, 19: 1, 1894.



Elastic fibers are stained a deep silky-brown color, connective tissue, a pale brown. If it is desirable to have only the elastic fibers stained, wash for a few seconds in a 1 per cent hydrochloric acid alcohol before washing in water. The nuclei can be brought out by staining in Unna's polychrome methylene blue solution after washing the sections in water.

#### IV. Mucin

Mucin, the homogeneous intercellular substance secreted by the fibroblast under certain conditions, has certain chemical properties in consequence of which it can be stained differentially. It is colored as a rule more or less intensely blue by alum hematoxylin after any fixative and by methylene blue in the eosin or phloxin methylene blue staining method after fixation in Zenker's fluid. The best of the differential stains are the following:

1. **Hoyer's<sup>13</sup> Thionin Method.** Fix tissues in a concentrated aqueous solution of corrosive sublimate for two to eight hours. Wash and dehydrate in frequent changes of 95 per cent alcohol. Do not remove the mercury precipitate with iodine. Imbed in paraffin in the usual way.

(1) Place sections in a 5 per cent aqueous solution of corrosive sublimate for three to five minutes.

(2) Wash off in alcohol or water.

(3) Stain in a dilute solution of thionin (two drops of a saturated aqueous solution, made by the aid of heat, to each 5 c.c. of water) for five to fifteen minutes.

(4) Wash in 95 per cent alcohol followed by absolute.

(5) Clear in xylol and mount in xylol balsam.

The nuclei are stained blue, mucin, mast cell granules, cartilage and amyloid, red. The mucin appears of a bright red if the sections after step (3) are examined after mounting in water or glycerin. According to Herxheimer toluidin blue can be used in place of thionin and gives more permanent preparations.

2. **Mayer's<sup>14</sup> Mucihematein Method.** Fix tissues in absolute alcohol, imbed in paraffin.

(1) Stain sections in one of the following solutions for five to ten minutes:

(a) Hematein .....	0.2 gm.
Aluminum chloride.....	0.1 gm.
Glycerin .....	40.0 c.c.
Water .....	60.0 c.c.

<sup>13</sup> Hoyer, H. *Arch. f. mikroskop. Anat.*, vol. 36.

<sup>14</sup> Mayer, P. *Arch. f. mikroskop. Anat.*, vol. 36.

Dissolve the hematein in the glycerin and add the other ingredients:

(b) Hematein .....	0.2 gm.
Aluminum chloride .....	0.1 gm.
70 per cent alcohol .....	70.0 c.c.
Nitric acid .....	1 to 2 drops.

The first solution stains quicker and better but the alcoholic solution is preferable if the mucin swells much.

- (2) Wash in water.
- (3) Dehydrate in 95 per cent alcohol followed by absolute.
- (4) Clear in xylol and mount in xylol balsam.

Mucin appears blue, the other tissue elements, colorless. The sections can be stained with carmine before using the hematein solution if desired.

### 3. Mayer's<sup>15</sup> Mucicarmine Method.

Fixation in absolute alcohol. Paraffin sections.

- (1) Stain five to ten minutes in the following solution diluted for use 1 to 10 with water:

Carmine .....	1 gm.
Aluminum chloride (dry) .....	0.5 gm.
Water .....	2.0 c.c.

Heat over a flame for two minutes until the solution appears dark colored. Gradually add 100 c.c. of 50 per cent alcohol, stirring constantly until the mixture is dissolved. Filter after twenty-four hours. The solution keeps well.

- (2) Wash in water, dehydrate in alcohol, clear and mount in xylol balsam.

The mucin is colored red. A preliminary staining with alum hematoxylin is advisable. If the nuclei are stained red it indicates that the staining solution is acid and must be neutralized by the addition of a few drops of a 1 per cent solution of bicarbonate of sodium.

## V. Amyloid

Amyloid is an abnormal intercellular product of the fibroblast, chemically related to the ground substance of cartilage. It occurs not only in the liver, spleen and other organs as the result of certain chronic infectious and other processes but occasionally in localized masses and rarely in the stroma of tumors. It has definite chemical and physical properties on which certain characteristic staining methods are based.

The original stain for amyloid was iodine which colors it mahogany brown both in gross and in frozen sections. Nowadays more use is made of certain aniline dyes which stain amyloid metachromatically. The great difficulty in the past has been to obtain permanent mounts of sections in which the amyloid was stained characteristically. This has now

<sup>15</sup> Mayer, P. *Mitt. Zool. Stat. Neapel*, vol. 12: 1896.

been rendered possible by Mayer's ingenious and seemingly impossible method. Sections stained several years ago by his method are as bright and sharp today as when originally prepared.

In examining microscopically metachromatic stains for amyloid it is important always to take the light from a white cloud in order to obtain a sharp differentiation in color. The light from the blue sky is worthless.

#### 1. Iodine Reaction for Amyloid.

- (1) Stain sections in a weak solution of iodine (Lugol's solution diluted until of a clear yellow color) for three minutes.
- (2) Wash in water.
- (3) Mount and examine in water or glycerin.

If the tissue reacts strongly alkaline, a condition which may result from post mortem decomposition, the color reaction with iodine will not take place. In such cases the tissue or the sections of it should be treated with dilute acetic acid before applying the test. The normal reaction of amyloid with iodine may be increased by treating the section after staining with dilute acetic acid.

#### 2. Iodine and Sulphuric Acid Reaction.

- (1) Stain quickly and lightly in dilute Lugol's solution.
- (2) Treat with sulphuric acid, either concentrated or dilute (1 to 5 per cent), on the slip or in the staining dish. Strong hydrochloric acid may be used in the same way.

The color of the amyloid will usually change at once or in a few minutes from red, through violet, to blue. Sometimes the color turns simply to a deeper brown.

#### 3. Langhans'<sup>16</sup> Method for Obtaining Permanent Mounts with Iodine.

- (1) Harden in alcohol and stain in Mayer's alcoholic carmine solution.
- (2) Stain sections in Lugol's solution five to ten minutes.
- (3) Dehydrate quickly in 1 part of tincture of iodine to 3 or 4 parts of absolute alcohol.
- (4) Clear and mount in oil of origanum.

The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. The staining in Lugol's solution may be omitted, as the tincture of iodine usually stains the amyloid sufficiently deeply.

#### 4. Reaction with Methyl-violet.

- (1) Stain frozen sections of fresh or of formaldehyde or alcohol fixed tissue in a 1 per cent aqueous solution of methyl-violet three to five minutes.
- (2) Wash in a 1 per cent aqueous solution of acetic acid.

<sup>16</sup> Langhans. *Arch. f. Path. Anat.*, vol. 121: 1891.

- (3) Wash thoroughly in water to remove all trace of acid.
- (4) Examine in water or in glycerin.

The stain will keep for some time if mounted in a saturated solution of acetate of potash or in levulose. Other methods are to stain in aniline-methyl-violet and to wash out in a 1 per cent solution of hydrochloric acid, or to stain in a strong solution of methyl-violet to which acetic acid is added, and to wash out in water. The amyloid is stained violet red, the tissue blue. Sections of tissues imbedded in celloidin will not give the reaction unless the celloidin is removed.

#### 5. Reaction with Iodine Green.

- (1) Stain fresh or hardened sections in a 0.3 per cent aqueous solution of iodine-green for twenty-four hours.
- (2) Wash in water.
- (3) Mount in water or glycerin.

Amyloid, violet red; tissue, green. Stilling claims that the reaction is surer than with methyl-violet.

#### 6. Reaction with Bismarck Brown and Methyl-violet (Birch-Hirschfeld).<sup>17</sup>

- (1) Stain in a 2 per cent alcoholic solution of Bismarck brown for five minutes.
  - (2) Wash in absolute alcohol.
  - (3) Wash in distilled water ten minutes.
  - (4) Stain in a 2 per cent aqueous solution of methyl-violet five to ten minutes.
  - (5) Wash in dilute acetic acid solution.
  - (6) Wash thoroughly in tap water.
  - (7) Mount in levulose.
- Amyloid, red; tissue, brown.

#### 7. Mayer's Stain for Amyloid.

- (1) Transfer paraffin sections without previous treatment directly from the knife to a warmed (40°C.) 0.5 per cent aqueous solution of methyl- or gentian-violet for five to ten minutes.
- (2) Wash in water and differentiate in a 1 per cent solution of acetic acid for ten to fifteen minutes.
- (3) Wash thoroughly in water.
- (4) Transfer to half saturated aqueous solution of alum. Wash off in water.
- (5) Transfer sections to slip and allow the water to evaporate.
- (6) Remove paraffin and clear with xylol. Mount in xylol balsam.

The same method can unquestionably be used with crystal violet and iodine green.

<sup>17</sup> Birch-Hirschfeld. Festschr. f. El. Wagner. Leipz., 1887.

## METHODS FOR PREPARING MUSCLE AND ELECTRIC ORGAN TISSUES

ULRIC DAHLGREN

Demonstrating general cellular character of muscle 420. Demonstrating special structural character of muscle 421. Identification of muscle substance by stains 424. Studying cell organs of contraction 425. Study of muscle in any stage of contraction 428. Study of cytoplasmic inclusions 431. Demonstrating cell membranes and sarcolemma and connective tissue attachments 431. Demonstrating other tissue elements associated with muscle cells 434. Study of electric tissues 434.

In considering the procedure necessary to make microscopic sections or other mounts or examinations of muscle tissue or suspected muscle tissue, the following objectives should be taken account of:

1. The general features, as cytoplasm, nucleus, cell wall, centrosomal structures, chromidia, Golgi apparatus, etc., all of which the muscle cell (or syncytium) shares with most other cells. The methods indicated for examining these structures or parts are the same that would be used for similar structures in most other cells, and the worker is referred to the portion on general cytology. These parts are usually overshadowed in our consideration by the cell organs of contraction.
2. General views of muscle.
3. The identification of muscle tissues and cells.
4. The cell organs of contraction, the myofibrils. These are the most important structures in the muscle cell and the most weight will be placed on a discussion of the methods used in studying them.
5. Technique of securing muscle in any stage of contraction or relaxation.
6. The myochondria or muscle fuel substance.
7. The cell membrane and sarcolemma and other outer envelopes of the muscle fiber and the connective tissue attachments and intercellular connectives of muscle cell.
8. Nerve connections or nerve plates, circulatory structures and other foreign structures associated with the muscle cell.
9. The electric cells of fishes (modified muscle cells).

### I. Methods for Demonstrating General Cellular Character of Muscle

See technique used in general cytology, Chapter V.

## II. Methods for Demonstrating Special Structural Character of Muscle

For this purpose we can use:

(a) Teased fresh preparations or total views of small animals such as *Entomostraca*. Such examination is valuable and enables one to use several methods of lighting, as transmitted light, reflected light, dark-field illumination and polarized light. Some of these, particularly the polarized light, permit very beautiful views of muscle activity and study of the isotropic and anisotropic areas during contraction and relaxation.

(b) Sections in paraffin or celloidin of large areas of muscle or body regions in which muscle is found, for the study of the general histology of the tissue and its surroundings. We have to deal here with the subject of muscle fixation, imbedding and staining.

In most cases muscle tissue contains large numbers of myofibrils, and under technical treatment this substance shows a strong tendency to harden and become brittle. Great care should therefore be exercised to employ the proper fixing fluid, as short a fixation as will serve, a rapid dehydration and, in the case of paraffin sections, as short a subjection to heat in the water bath at as low a temperature as possible. Otherwise two familiar artifacts, *pressure ridges*<sup>1</sup> and *section cracks*<sup>2</sup> will appear in embarrassing profusion.

Paraffin sections can be cut from  $1\mu$  or  $2\mu$  in thickness up to  $10\mu$  or  $12\mu$ . Section cracks are most likely to appear in the thicker sections. Celloidin sections are better for very general views than paraffin sections. Since the tissue does not have to endure the heat of imbedding in paraffin, we are not troubled by the *cracks* and *ridges*. An artifact more apt to occur in celloidin is the condition of *free ends*.<sup>3</sup>

<sup>1</sup>*Pressure Ridges*. In this artifact the knife edge, especially when dull, and encountering a tough substance in a softer paraffin, compresses the material, pushes it slightly forward and then suddenly cuts through it leaving a region of compressed and sometimes darker staining material behind it in the section. This usually leaves a series of such "ridges" in the section which may be misinterpreted.

<sup>2</sup>*Section Cracks*. This irregularity appears, usually, when a sharp knife edge encounters a hard and brittle substance, well imbedded, and as the section lifts from the block at the angle of the knife edge it cracks at regular intervals, either all the way through or only part way through the thickness of the section. Such cracks are very apparent in the finished and mounted section and their spacing is determined by the distance the knife can go before each crack occurs. In muscle tissue this artifact is much more likely to occur when the fibers are cut longitudinally from one end than when the knife approaches them from the side, while pressure ridges are more frequent when they are cut with the knife-edge parallel with the fiber.

<sup>3</sup>*Free Ends*. This artifact is most likely to occur in longitudinal sections of the

1. **Fresh Material.** *a. Teased Tissues.* For striated muscle, bits of vertebrate flesh or arthropod flesh may be used with some success. They should be teased with needles in normal salt or Ringer's solution. In life, under ordinary illumination, this method is not very successful but the individual myofibrils can be seen and the segmentation of each fibril into isotropic and anisotropic substances becomes quite apparent, especially with a strong illumination, much cut down by the substage diaphragm. Smooth fibrils (i.e., those without such segmentation) give poor results under such examination, the difference in refractive index between fibril substance and cytoplasm being so small during the life of the cell that the fibrils are seen with difficulty.

*b. Whole Animals.* A better way to see active muscle is to observe some small living animal, or part of an animal, in which the fibers are so isolated and the surrounding tissues so scarce and transparent that they can be seen through the whole body or member. Pelagic or fresh water *Entomostraca*, certain aquatic insect larvae for striated fibers, and very young small and thin leeches or other *Annelids* or other invertebrate larvae for non-striated fibers, serve this purpose best. Here again the striated muscle is the more easily observed because the difference of refractive index is greater between the isotropic and anisotropic substances of the fibril than between the fibril and its surrounding cell structures.

When polarized light is used, the above examinations of the fibers as a whole becomes much more significant. In this case, as the term implies, the anisotropic and isotropic substances of striated muscle become as sharply differentiated as black from white because the polarized light will penetrate one but not the other. At such times the movements and relations of the two regions in the fiber can be plainly seen or even photographed. Such movements are quick but may be slowed down by cooling.

2. **Prepared Materials.** *a. Macerated Tissues.* Better visibility under ordinary light is shown by muscle fibers that have been fixed and dissociated and then examined as they are or stained. The fixing process can be made to perform the dissociation as well when fixatives that contain a connective tissue solvent are used. A young trout (3-20 cm. or more), fixed in Bouin's fluid for ten days to three weeks, will yield bits of flesh that can be shaken in a test-tube of water or 70 per cent alcohol so that large numbers of individual fibers will be available. Sublimic-acetic will do the same, not quite so satisfactorily but still adequately.

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muscle fibers. Since the plane of section can hardly ever be made to coincide exactly with the myofibrils, these latter will have many cut ends in the length of the fiber and such ends are very apt to become free at their tips and stick up or lie at an angle to the course of the fibers. This is not difficult to deal with, however, as by focussing slightly down these free ends disappear.

In both cases it is the acetic acid that dissolves the connective tissue. Flemming's fluid and Zenker's fluid are not so satisfactory for this purpose because the chromic compounds work against the acetic acid. Weak osmic acid ( $\frac{1}{10}$  of 1 per cent in distilled water) alone will dissociate and fix muscle fibers in an exquisite manner in a few days. With 1 per cent of acetic acid it will dissociate quicker with almost as fine a fixation. In the first case small masses should be used (not over a few millimeters in thickness). In the latter twice that thickness is possible. Ranvier's alcohol ( $33\frac{1}{3}$  per cent) will do this work with rather poorer fixation. Smooth muscle from the mammalian bladder is more resistant and from 10 per cent to 20 per cent nitric acid is required for dissociation. This method is applicable for class purposes.

*b. Sectioned Material.* Frozen sections of striated muscle may be made and observed much as the fresh teased or entire specimens were. Polarized light and vital stains can be utilized. The method is not so valuable for smooth muscle. The sections are of greatest use when made parallel to the course of the fibers and a little thicker than their diameter. There is no point, in most cases, of using transections of frozen material.

Thicker sections in paraffin or celloidin or the combined method are of great value in giving general views of the superficial structure of both striated and non-striated muscle and in showing the relations of the fibers to the connective tissues, blood vessels, nerve fibers, tracheae, etc., which surround them. For this purpose Zenker's fluid, Bouin's fluid, and many other common fixatives are well fitted. When the connective tissues formed by or associated with the muscle cells are to be a special point, strong acids are to be avoided, especially hydrochloric, nitric and acetic. In these cases pure corrosive sublimate solutions or Zenker and Bouin without the acetic may be used with success. Müller's fluid is most excellent for this purpose when the nuclei and finer details of the cell are not to be considered. This fluid dissolves the chromatin but not the nuclear membrane so that nuclei appear as homogeneous spheres and it sometimes injures the striation. Subsequent staining with Delafield's hematoxylin and eosin should be employed. Iron hematoxylin is not advised with this fixation.

Van Gieson's picric acid and acid fuchsin is very good, showing as it does the muscle substance yellow and connective tissues red, but the first-mentioned stain seems most suited to general work. In general too great reliance on a variety of stains, especially highly complicated special stains, should be discouraged. The continued and repeated use of two or three standard stains with varied timing and concentration may



be followed with profit by the use of some of the more special ones. The same may be said of fixatives.

### III. The Identification of Muscle Substance by Stains

Striated muscle can be identified at a glance, either stained or unstained, except in very unusual cases when the fixation has been so poor that the striation cannot be seen. On the other hand smooth muscle is often found in positions where its location is unfamiliar or unusual and when it must be distinguished from certain connective tissues. Both act in a tensile connective capacity, both have specific cell organs which are fibrils; in fact one and the same cell may in some cases form myofibrils in one part of its cytoplasm and connective tissue fibrils in another. Myofibrils are always contained in the cytoplasm, though sometimes very near its outer surface. Connective tissue fibrils are often on and outside the surface. When inside, the question often arises as to whether it is a contractile fibril (muscle) or a non-contractile fibril (connective tissue). Such a question still stands in regard to the fibrils in the end cells associated with the luminous organs of the *Lampyridae* and has never yet been answered. To see a fibril contract in life under the microscope is definite and positive proof of its muscular nature. To see it fail to contract is a dubious negative proof. To find it in one case shorter than in others is an unsatisfying proof. Staining is sometimes the most practical method of making a determination but it should be employed with a certain reserve. When the whole body of small animals or a heterogeneous part of the body of larger animals is sectioned and stained uniformly the method of comparing the questionable fibrils with known muscle tissue is often decisive and is a means often employed.

Most muscle tissues are easily stained with the acid dyes, especially eosin. This alone, however, is not sufficient to distinguish them from many connective tissues; for this reason mixtures have been devised that will stain them differentially. Several such stains should be used with care in making a determination.

1. **Retterer's Stain.** Retterer has devised an excellent and simple stain for differentiating muscle tissue (this is for smooth muscle but it also works well with striated forms) from connective tissues in general. He fixes in 10 volumes of 80 per cent alcohol to which 1 volume of formic acid has been added and then stains in alum carmine. The muscle should show a light red stain while all connective tissues remain unstained.

2. **Van Gieson's Stain.** Van Gieson has formulated a stain for sections, composed of picric acid and acid fuchsin, in which muscle stains

yellow and connective tissue red. Others have varied his formula with success which shows that in some cases there must be a greater proportion of the acid fuchsin or the connective tissue will also be yellow, while in other cases there must be a lesser proportion of the fuchsin or both muscle and connective tissue will be red.

3. **Picro-nigrosine.** Picro-nigrosine is a short and rapid stain for distinguishing muscle from connective tissue. Dissolve nigrosine in a saturated solution of picric acid in water. Fixation should be in alcohol or Bouin's fluid. The muscle will be yellow, the connective tissue dark.

4. **Unna's Orcein.** Unna's orcein method after sublimate fixation is good for this purpose with some practice. Stain for twenty-four hours in orcein 1 gm., wasserblau 0.25 gm., alcohol 60 c.c., glycerin 10 c.c., water 30 c.c.; wash in 70 per cent alcohol, dehydrate, clear and mount in balsam. In observing muscle with this stain one must distinguish muscle by comparison with known stains until the mixed purplish color has become familiar. Collagenous and white connective tissues are decidedly blue and elastic connective tissues decidedly red.

The above methods are for use with sections only and for comparisons of the whole muscle cell and its myofibril content of muscle substance with white or elastic or other connective tissue fibrils. (See also p. 433.)

#### IV. Methods of Studying the Cell Organs of Contraction

1. **The Myofibrils.** The study of the myofibrils is the most important feature of muscle preparation and investigation. These structures are the specific cell organs of contraction. They are inside the cytoplasm, are formed and controlled by the cytoplasm and have a higher index of refraction and a greater tensile strength than the cytoplasm. They are always very small, which permits of adequate penetration of reagents and makes shrinkage almost imperceptible.

a. *Fixation.* The myofibrils are easily fixed by almost any fixative, do not perceptibly shrink and tend to stain the same no matter what the fixative. In the different stages of their contractile activity they undergo large chemical changes which are strongly indicated by their staining reactions. It is desirable to study the myofibrils in both longisection and transection and in the former case they should almost always be studied in sections thin enough to contain not more than one or two fibrils between the two planes of the section. Celloidin alone is usually not indicated. Paraffin and the celloidin-paraffin method of double imbedding usually provide the best results. Both a basic and an acid stain should be applied to the specimen in most cases, because the isotropic

substance has a strong affinity for the acid stains such as eosin, while the anisotropic material absorbs and is stained by the basic dyes like hematoxylin or safranin. When the two substances are not separated so as to be locally distinguished, the double staining is still valuable to show areas of contraction.

*b. Staining.* A most important factor of muscle study is to distinguish between *absorption* or *retention* of the dye and staining by the dye in the myofibril. Owing to its denser nature the myofibril will absorb or retain in a purely physical way a much larger proportion of the dye than will the cytoplasm; also for the same reason, in striated muscle, the anisotropic substance will retain or absorb much more dye than will the isotropic. Again, the contracted areas of smooth muscle will absorb and retain more stain in this physical manner than the non-contracted. Therefore, in all regressive staining for careful study, the crude stain should be extracted until a real chemical staining remains which will identify the anisotropic substance or contracted area, rather than the absorbed or retained masses of dye that merely indicate its locality. For general class purposes the latter more crude condition is useful and shows very beautiful pictures. In using iron hematoxylin the above should be most carefully considered.

When chemically stained with safranin, gentian violet and other basic aniline dyes, the anisotropic areas and the contracted areas of smooth muscle present stronger pictures than with iron hematoxylin when properly extracted. In seeking to merely locate myofibrils and study their relations to the surrounding cytoplasm the acid dyes, such as eosin, acid fuchsin, etc., should be depended upon.

*c. Sectioning.* Paraffin sections alone should be used to study the longitudinal sections of the myofibrils in the fiber, while the celloidin-paraffin method will serve in the study of transections of the fibers for observing the relations of the fibrils to the cytoplasm and to each other. This is because the celloidin prevents the artifact called *tilting*<sup>4</sup> which often occurs with paraffin alone. It is much less likely to occur in very thin sections.

One of the best stains for studying the myofibrils is iron hematoxylin. It should be applied in a variety of ways as has been noted by Heidenhain and many other workers. With striated muscle the stain should

<sup>4</sup>A fourth artifact may be known by the name of *Tilting*. This occurs only in transections of muscle fibers and is very common there. In an exact transection the myofibrils should point exactly up toward the observer. When tilting occurs some or all of them have tipped over so that an oblique or even lateral view is presented. This artifact takes place in paraffin sections much oftener than in celloidin or paraffin-celloidin sections or regular celloidin sections and in thick sections oftener than in thin ones.

first be stopped with only a sufficient decolorization to leave the *M* or *Q* stripes, the *N* stripe and the *Z* stripe a deep black while the isotropic substance is clear. This is the infiltration stage and is most useful for general views and for most class work.

In another slide the stain should then be extracted until the *M* stripe is gray, the *N* stripe has cleared (is usually more bluish) and the *Z* stripe shows as a sharp black line, in relaxed muscle. If contracted the *M* stripe will be yellowish and thinner and the *Z* stripe will be a broad segment in which there is added to the Krause's membrane the substance of adjoining *M* stripes which have obliterated the *N* stripe and masked the *Z* stripe (Krause's membrane) so that the dark bands of the muscle appear to have been moved from the *M* position to the *Z* position leaving the *M* position clear and unstained, save for a dim line in some forms. In excessive contraction the *Z* bands have been observed to be one-eighteenth the length of the resting units and so closely approximated that they touched, thus making the substance an apparently almost solid mass. In certain post-contraction stages the staining power appears to be lost, leaving only one band present, the *Z* line. We will omit here the various intermediate stages and appearances which belong more to a discussion of muscle histology than to an exposition of technique.

Some of the other valuable stains for the striated myofibrils are Mallory's phosphotungstic acid hematoxylin and Mallory's aniline blue connective tissue stain after fixation in Zenker's fluid (p. 558).

In smooth muscle myofibrils are also present. They respond on the whole to fixatives and stains much as the myofibrils of striated muscle do. Zenker's and Flemming's strong mixture are among the best fixatives. Iron hematoxylin stands first as a stain. The fibrils are not divided into contractile units (sarcous elements) as in striated fibers, but contract in irregularly placed regions called by McGill "contraction nodes" in which the fibrils shorten and thicken and change in their chemical make up so that they are evidently denser and retain dyes both by infiltration (or absorption) and by chemical union (staining).

**2. Krause's Membrane.** This membrane divides the sarcous elements (contractile units) of striated muscle from each other. It is of a higher refractive index than any other cytoplasmic element in the muscle cell and takes the black of iron hematoxylin strongly. The myofibrils pass at right angles through it and the membrane extends out into the free cytoplasm from side to side of the fiber where it joins with the sarcolemma. It may be stained at times by Bielschowsky's silver stain. It is non-contractile but may be expanded and made wavy by fixation with boiling water. When the muscle contracts its non-extensile char-

acter causes the cytoplasm to bulge out between the points of attachment. By treatment with 98 per cent alcohol the muscle fiber becomes brittle and tends to break all the myofibrils at the intersection with Krause's membrane into a number of transverse discs. Weak chromic acid, on the contrary, may be used to rupture Krause's membrane between the myofibrils and thus free them into longitudinal elements.

The N stripe during relaxation is stained bluish gray by iron hematoxylin. It may also be demonstrated by the Golgi silver stain. This structure lies in the cytoplasm and is not a part of the myofibrils but is mentioned here because it is very often erroneously considered to be a secondary anisotropic region of the myofibrils, which it is not. During contraction the stainability and increased density of this region cause this structure to become invisible.

## V. Method for the Study of Muscle in Any Stage of Contraction

1. **Striated Muscle.** To secure any definite stage of contraction in a muscle preparation has proved a more difficult thing than might be thought. Though an animal be dead the muscle tissue may still show reaction to stimulation. The mode of tissue death often determines the physiological picture presented by the stained slide. The length of the muscle when tissue death occurs does not always establish the physiological stage of contraction shown, as a myofibril in such stage may be stretched and still retain the picture of physiological contraction. Ordinary preparations, where the tissue from an animal killed by decapitation is thrown into the fixative, will show in nearly all cases a stage of weak contraction which is sometimes varied by occasional areas of stronger contraction.

One of the best animals from which to secure striated muscle in all stages of contraction is the large larva of *Corydalis cornutis* which can be found in quantities under the larger flat stones of most of our medium-sized running streams. These animals are tough but soft, and very muscular. The skin can be very easily cut by the microtome knife and does not have to be removed.

One animal should be chloroformed until entirely lax and dead. It is then pinned out straight on a cork sheet and its body cavity filled with Flemming's or Bouin's fluid by injection with a hypodermic syringe. After five to ten minutes, with a sharp pair of scissors cut along each side of the body so that the upper and lower body walls can be readily separated, the internal organs and fat-bodies removed and the two principal muscle masses further fixed and treated for sectioning. When cut and stained such muscle should show very

little contraction. Fixation with boiling water followed by Bouin's will also show very clear and bright pictures but the z line will be slightly swollen and waved.

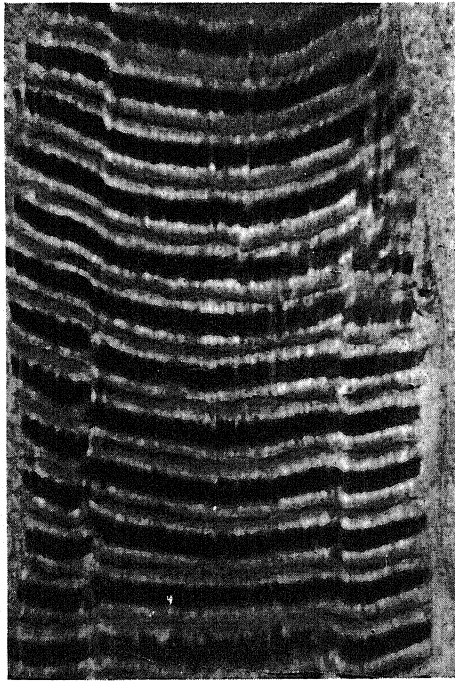


FIG. 1. Longitudinal section of a bit of body muscle of *Corydalis cornutus*. Relaxed state. Fixed in Bouin's fluid. Stained in iron hematoxylin. Fixed by body injection of fixing fluid. Prepared and photographed by C. S. Shoup.

Pin out another live animal in the same way and hook the copper wire poles of 2 dry cells of  $1\frac{1}{2}$  volts each with an inductorium in the circuit. Draw out the induction coil to between 3 and 4 cm. so that a tetanic current will pass through the animal's body when the current is turned on. Then insert the hypodermic needle into the body cavity and, just after the current connection is made, flood the body cavity with Flemming's or Bouin's fluid, keep the electric current running for about thirty seconds and then cut the animal open as before and complete the fixation and hardening, etc., and cut sections.

Muscle should be found in such sections in all stages of contraction. Some muscles with contraction waves will be found in which the process can be followed from inception to finish; also fatigued muscle that has relaxed. In the contracted portions, sarcoous elements will appear that have contracted to  $\frac{1}{18}$  of the length of those in resting fibrils (Figs. 2

and 3). This is more than a normal contraction and in consequence some fibers will be found ruptured. Another animal should be treated in the same way with a weaker current for comparison.



FIG. 2. Longitudinal section of a bit of body muscle of *Corydalis cornutus*. Excessive contraction under 3 volt current from an inductorium. Coil extended 4 cm. Same scale as Figure 1. Same fixation and stain as Figure 1. Prepared and photographed by C. S. Shoup.

**2. Smooth Muscle.** Smooth muscle may be secured in different stages of contraction and showing contraction waves or areas by simpler means. A bit of freshly killed cat's intestine is cut out and exposed to the cooler air of the room or rubbed with the handle of a scalpel. Sections will show many contraction areas. Or the animal may be chloroformed to death and, just as rigor mortis is setting in, bits of bladder or intestine quickly fixed will show fully extended smooth fibers.

The foot and valve muscles of marine *Pelecypod* mollusks may be completely relaxed by an excess of calcium salts in the sea water. This same muscle, freshly cut out, will be contracted. Other methods remain to be devised for these and other animals, especially some of the invertebrate forms.

## VI. Methods for the Study of Cytoplasmic Inclusions

The cytoplasm of muscle cells contains in normal tissues a large but varying amount of material which has been stored there by its metabolic activity against the needs of its contractile activities. This material is usually in a fluid condition but in some muscles it may appear as a solid. When visible, it ranges in appearance from a reticulated precipitate to small granules and up to large granules with a definite form and location. The best place to see them is in the thoracic wing muscles of the solitary hornet *Sphecius*, and probably in other allied forms. In *Hydrophilus* these bodies, which may be termed myochondria, are large and visible but not arranged regularly. We have no differential stain as yet to identify this material but when in granules it can be adequately located by fixing the muscle in sublimate acetic or chromacetic-formalin, cutting thin sections and staining with iron hematoxylin. The sections should be not more than  $5\mu$  thick and the iron hematoxylin should be withdrawn until the M stripe is decolorized and only a faint image of the Z line can be seen in the large myofibrils. At this point the myochondria will remain a deep black with absorbed hematoxylin and the decolorizer should be quickly washed out. A counterstain such as eosin, may or may not be valuable.

The regular arrangement of the myochondria in *Sphecius* is so marked, two between each adjoining pair of sarcous elements, that most observers are easily deceived into believing that they are looking at the black M stripes when they are only looking at the transverse rows of myochondria in the cytoplasm between the myofibrils. The best way to see the difference easily is to observe the thinned edge of a wedge-shaped section which often occurs when the paraffin block is a little too large and hard. Here the loose end artifact displaces some of the fibrils in longitudinal sections. Also in thin transections the arrangement becomes plainer.

In the heart muscle of *Homarus* certain spike-shaped or tack-shaped bodies in the cytoplasm are rendered visible by several stains, the best of which is Delafield's hematoxylin.

In most muscle fibers the cement substance that binds the myofibrils into groups has not been differentially stained except as to degree.

## VII. Methods for Demonstrating Cell Membranes and Sarcolemma and Connective Tissue Attachments

A sarcolemma or thin outer covering of connective tissue has been demonstrated in all larger muscle cells. This ranges from a covering of



thin connective tissue secreted from regular connective tissue cells that are in the neighborhood, through membranes partial or complete, secreted by the muscle cell's own cytoplasm, to muscle cells in masses

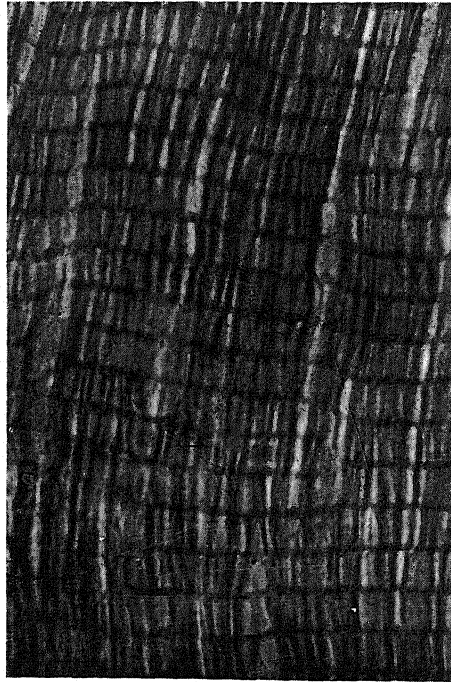


FIG. 3. Longitudinal section of a bit of body muscle of *Corydalis cornutus*. Just relaxed after contraction under electric stimulation. Same fixation and stain as Figure 1. Same scale as Figure 1. Prepared and photographed by C. S. Shoup.

where this outer cytoplasmic activity is confined to the formation of strands of connective substance which serve to bind spindle-shaped cells together and to take part of the contraction strain that may come on them. The technique of demonstrating these outer coverings is varied and has developed from numerous scientific disputes as to whether a sarcolemma was present or not, and if so whether it was secreted from the muscle cell or from surrounding connective tissue cells. The following techniques have been devised to meet these cases, in which the writer does not enter into the merits of the disputes as to the origin of the structures involved:

1. **Striated Muscle.** To demonstrate the sarcolemma of striated muscle in vertebrates Ranvier throws a live frog into boiling water. Muscle fibers in the thighs are ruptured and the ends are retracted

leaving the sarcolemma intact and passing between the ruptured ends where it is visible in the unstained specimens teased out in salt solution. Solger demonstrates the same structure by teasing fresh striated muscle in a saturated solution of ammonium carbonate under the microscope. Thin transections of muscle stained in iron hematoxylin and eosin, after Zenker and Flemming's fixation, show the thin line of the sarcolemma taking the eosin, if sufficiently decolorized after the hematoxylin, but retaining a gray to black color if still infiltrated with the hematoxylin.

**2. Smooth Muscle.** In smooth muscle and all muscle masses containing spindle-shaped cells, the connective tissue fibrils that unite the cells of the mass are secreted by the ectoplasm of the muscle cell itself. These fibrils are flat and form a reticulum in the smooth muscle of mammals. They may best be seen in sections of the bladder wall hardened for seven to ten days in Flemming's strong mixture and stained in iron hematoxylin, a long stain (twelve to sixteen hours) with a decolorization just sufficient to leave the muscle cells clear yellow, when the connective tissue fibrils will appear from dark gray to black. Bielschowsky's silver stain will, in the majority of cases, show the muscle cells light yellow (unstained) and the connective tissue fibrils light to dark brown. Van Gieson's picric acid fuchsin stains the connective fibrils light pink, and is unsuitable owing to their delicate nature. An especially good demonstration of such connective tissue fibrils is to be seen in transection of the aorta or other large arteries leaving the heart of cephalopod mollusks. Here the blunt ends of the striated circular muscle cells show these connective tissue fibrils radiating to points of attachment on the sides of the adjoining muscle fibers and only an iron hematoxylin stain is necessary to demonstrate them.

Where muscle fibers are attached to bone, cartilage, shell etc., it is sometimes difficult to decide where the myofibril of the muscle fibers ends and an intervening connective fibril begins. Usually an epithelial cell (mollusk, insect etc.) or a connective tissue cell (vertebrate) intervenes and the myofibrils appear to be continuous with connective tissue fibrils belonging to these two kinds of cells. A chemical differentiation may best be shown in these cases by Mallory's aniline blue stain (p. 405).

In addition to these white connective tissue fibrils and reticular structures a network of very fine elastic fibrils is found in some muscle masses, running around and between the muscle cells. To see these, use some good elastic tissue stain such as Unna's methylene blue and acid fuchsin after fixation with Flemming's strong mixture. Stain sections five to seven minutes in polychrome methylene blue, wash in water and then stain them for ten to fifteen minutes in an 0.5 per cent solution of acid

fuchsin plus 33 per cent of Grübler's tannin mixture. Elastic fibrils should be the only blue fibrils in this preparation.

The heart muscle of vertebrates shows at regular intervals in the fibers a series of lines of cell attachment, the intercalated disks or cement lines. Such a line occupies a position in the striation corresponding to the location of Krause's membrane, from which it may be derived, and is best stained with Mallory's phosphotungstic hematoxylin after a Zenker fixation. It is not easily stained and may not be clearly visible in all parts of the section.

### VIII. Methods for Demonstrating Other Tissue Elements Associated with Muscle Cells

For this technique see page 432.

### IX. Methods for the Study of Electric Tissues

These tissues, known in only seven types of fishes, are derived from muscle cells and one characteristic is that they retain in varying degrees the firm, easily fixed myofibril masses found in muscle. In the more highly specialized electric organs the electroplaxes have least of this substance and are more difficult to secure good preparations from, shrinkage being the factor to be guarded against. The cytoplasm, in which such myofibrils as are present lie, is peculiarly soft and contains much water-soluble material that passes out during the technical processes. Consequently the tissues may be divided into two groups, those with the larger amount of residual myofibrillation, enough to make successful preparations probable, as *Astroscopus*, *Raja* and *Mormyrus*, and those which show but little or no residual myofibrillation, as *Malopterurus*, *Electrophorus*, *Torpedo*, and *Gymnarchus*. The following two methods of fixation and imbedding have been found to be the only ones so far that will preserve the general form of the units of the second group in anything like their natural form and size. For these one of the following two methods should be used:

(1) Place bits of tissue, a centimeter in thickness, in Flemming's strong mixture and allow them to remain for three or more weeks with one change after about twenty-four hours. Wash in running tap water for six hours and run up slowly in the alcohols. Imbed carefully in celloidin, first a thin solution and then the strong mixture. Harden the block in pure chloroform until dense, change to a mixture of two parts chloroform and one of cedar oil and cut sections as thin as convenient. Stain with iron hematoxylin and mount. Or, with but slight further shrinkage, the block of celloidin may be cut off the carrier and imbedded

in paraffin and thinner sections cut, floated out on warm water on the slip, cooled and dried fast to it and an almost equally unshrunk picture secured. These latter sections can also be stained by safranin and any other dyes that can be used after Flemming's mixture.

(2) This method depends on formalin. Bits of the tissue may be fixed either in 10 per cent formalin, or in any fixative like substance or picric acid containing this amount of formalin, for three or more days and then imbedded and cut as above in celloidin. This will permit of other staining but the double imbedded specimens will suffer somewhat more shrinkage than the Flemming's material.

These two methods must be followed in the second group and may be followed in the first group of electric tissues with advantage. All other ordinary fixing methods may be employed with the first group but the imbedding must be done with care. Owing to the presence of a semi-permeable membrane on all electroplaxes osmotic action is strong and especially in the case of the skate (*Raja*) great distortion produced by this condition is common. Often in electric tissue from this fish the membrane which covers the entire electroplax will swell up like a balloon with the cytoplasm and myoid substance sticking to the anterior or the posterior curvature.

No specific stains have been discovered for electric tissue. The myoid substance stains much like muscle, showing only a little less affinity for eosin. The large nerve connections are easily stained with nitrate of silver by Bielschowsky's or Golgi's silver methods, perhaps more easily than those found in muscle. The electric connective tissue found between the electroplaxes stains much like lax white connective tissue. Embryonic electric tissues act much as do other embryonic tissues. The peculiar softness and soluble contents of the electric elements appear at an early stage, however.

The principal cytoplasmic inclusions of the electroplax, as in muscle, seem to be an energy-storing substance that is most often fluid but in some cases assumes the form of solid granules. This substance is solidified by the osmic acid in Flemming's fluid and by formalin treatment, as is implied above. When overtreated with Flemming's strong mixture it becomes blackened, so that one should, in using such a section, refer to those electroplaxes that lie just inside the more heavily blackened layers. The central portions are often useless because the substance has been dissolved out in the preparation. Iron hematoxylin or safranin are good stains.

In the *Torpedo* electric organ that has been carefully prepared by even ordinary methods and cut in paraffin the thickness of the electroplaxes is often less than the diameter of the nuclei, which then bulge out

the upper and lower membranes. In carelessly prepared specimens so sectioned the upper and lower membranes are all that is left and one must be an expert to say whether any given nucleus is within one or between two electroplaxes.

Much work has been done on electric tissues by special methods. They are easily dissociated and mounted whole to advantage by any dissociation methods. To see the myo-striation in *Astroscopus*, *Raja* and *Mormyrus*, dissociate in 15 per cent nitric acid and mount unstained in glycerine-jelly. The nerve endings have been successfully studied by the silver methods of Golgi and Bielschowsky. Also with methylene blue by Retzius. Electrochondria or the solid form of the stored fuel material has been demonstrated in *Mormyrus* and *Malopterurus* by staining with iron hematoxylin after Flemming's fixation. These granules, however, appear as almost unstained or yellowish bodies in the cytoplasm. The "rods" in the electroplax are stained black by absorbed iron hematoxylin and gray by further extraction (the true stain). They may be differentially stained clear red on a purplish ground by acid fuchsin in Mallory's connective tissue stain as shown by Hughes.

## NEUROLOGICAL TECHNIQUE

W. H. F. ADDISON

Removal of nervous tissues from body 437. Isolation of nerve cells, and of nerve fibers 439. Methods of preservation 441. Macroscopic staining of gray and white substance 442. General histologic structure 443. Shape and relationship of neurons 445. Special structures in cell bodies 449. Normal myelin sheath 469. Degenerating myelin sheath 475. Nerve endings 476.

The chief methods devised for the study of special features in the nervous tissues are outlined in this chapter, as well as some of the general methods which have been found by experience to be useful in this field.

In no field of technique, perhaps, are minor modifications so necessary and therefore so numerous as in this one. Many different factors, such as species of animal, age, myelination and functional condition, as well as other imperfectly known factors, demand a variation in the procedure. The result is that one has to rely on the experience gained by repeated trials.

If only a general survey of the tissue is desired, certain general histological methods are useful, but if a special study of relationship of neurons, growth, or cytologic details, then special appropriate methods are demanded.

### I. Removal of Nervous Tissues from Body

In the preparation of the nervous tissues for study the first step is the careful removal of the material from the body into the fixing fluid. In the case of small embryos the whole specimen or a part of it, such as the head, is usually prepared entire. In large fetuses or the young of small animals the top and sides of the brain are exposed by removal of the cranium but the brain may be allowed to remain in the base of the cranium until after fixation and washing. In larger animals the brain is usually removed by freeing it on all sides. This is an important step in securing good results. The general rule is not to handle, press on, or stretch the part desired, but to dissect and cut away surrounding structures until the part is exposed.

In the removal of the central nervous system, one needs bone forceps of several sizes, and sometimes fine-toothed saws, in addition to the usual dissecting instruments. In taking out the human brain the ordinary postmortem technique is followed. In the case of smaller animals, smaller

sizes of instruments must be used. Thus in removing the brain of an albino rat, it is advisable to use manicure forceps, and fine-pointed scissors. After complete evisceration, or thorough bleeding of the animal, the skin of the head and the muscles of the occipital region are cleared away to the bone. One point of the fine bone forceps is inserted gently between the atlas and one of the occipital condyles, keeping the inner point of the forceps close to the latter, and with pressure the condyle is broken. Then the other condyle is similarly cut. The occipital bone can then be turned upward and the dorsal surface of the medulla and part of the cerebellum exposed. It is usually advantageous also to cut the atlas on both sides and remove the upper part thus separated. Then by cutting forward with the bone forceps on each side, the lateral and dorsal aspects of the cranium are quickly taken off in small pieces. In young animals the bones readily separate along their sutures. The chief difficulties are with the parafloccular lobes of the cerebellum and with the olfactory bulbs. Each of the former lies in a lateral bony recess which has a narrowed connection with the cranial cavity. The dorsal margin of this recess must be carefully chipped away to free the paraflocculus. The olfactory bulbs at the nasal end of the brain are held closely to the bony floor by the olfactory nerves which traverse the openings in the cribriform plate, and it is best to cut these nerves last of all. The membranes over and between the two bulbs should be pulled up gently, and removed so that the margins of the bulbs are entirely free.

The brain is removed from the cranium by holding the head of the animal with its nose pointed downward and with fine scissors cutting, at the tip of the calamus scriptorius, the junction of medulla with spinal cord and then the most posterior cranial nerves. As the weight of the medulla, aided by gentle pressure with the end of the forceps, causes it to fall away from the bone when these nerves are cut, the more anterior nerves come into view and are cut through in succession with fine-pointed scissors. Finally only the olfactory nerves remain, and these are cut across, and the brain allowed to drop into the dish of fixing fluid and to rest on the layer of absorbent cotton which covers the bottom of the dish.

If only a small portion of the brain is to be taken, the region around the part desired should be well exposed. Then with a safety razor blade, preferably moistened with the fixing fluid, cuts should be made completely through the brain on both sides of the part to be taken. Cut any nerves or membranes holding the part and then lift this on a spatula of appropriate size into the fixing fluid, or let the tissue fall into the fluid directly. Where it is desirable to preserve the entire brain

more quickly, the fixing fluid may be injected through the heart or the internal carotid artery. The brain is removed from the cranium later, and is usually placed in more of the same fixing fluid.

When peripheral nerves are removed, these should not be stretched or pressed with forceps. After the muscles have been separated to expose the nerve a piece of filter paper is laid on the part desired and the nerve cut across at both ends. Raise first one end of the paper cautiously, the moist nerve adheres to it, and the connective tissue fibers below the nerve can be cut with scissors and the nerve liberated from its surroundings. The paper is then placed with the nerve downward, on the surface of the fixing fluid. In the case of nerve trunks of large animals or of human nerve trunks, a glass rod or stiff bristle may be laid alongside the nerve and the nerve tied with soft string to the ends of the rod. Then the nerve is cut beyond the ligatures. The glass rod and the attached nerve are then stood up in the bottle of fixing fluid, so that the nerve is practically surrounded by the fixing fluid, and is not in contact with the inside of the bottle.

## II. Isolation of Nerve Cell Bodies and of Nerve Fibers

A rapid but crude method of viewing cell bodies, with considerable lengths of their processes attached, is to make a film preparation, as in examining blood. A small piece (1 to 2 mm. across) of gray matter of the ventral horn of fresh spinal cord preferably of a large animal is squeezed flat between two cover slips, and these are then slid apart. The film dries quickly in air, or may be dipped in 95 per cent alcohol for a few seconds and allowed to dry. The films are then stained with 1 per cent aqueous methylene blue or other basic aniline dye, for two to three minutes, washed in water, dried and mounted.

A more careful method is to place small pieces of fresh gray matter in a dissociating fluid, as Ranvier's  $\frac{1}{3}$  alcohol, or Gage's formaldehyde dissociator. The latter is made of formol (full strength) 2 c.c., with normal physiological solution 1000 c.c., and should act for two to three days. Large cells, as the multipolar cells of the ventral horn, may be dissected out under the binocular microscope. When the cells are partially freed by careful teasing, the pieces of tissue should be stained, as by carmine or picrocarmine, or by a dilute aniline dye. Excellent preparations are often to be obtained in this way.

To examine nerve fibers in the fresh condition a small piece of nerve just taken from the body is placed on the slip, and one end of the piece is separated into many little strands by pulling it apart with sharp-pointed needles, and by stroking the little bundles lengthwise with the



point of the needle. The use of a dissecting microscope for these manipulations is very helpful. To examine with a microscope merely add a drop of physiological salt solution and cover. The myelin readily changes its arrangement, forming irregular globules and droplets of varying sizes. This occurs on the addition of water, on pressure, and as a post-mortem change in the body.

To fix nerves intended for the isolation of individual fibers, 1 per cent osmic acid or 10 per cent formol may be used. The former is best for the demonstration of nodes of Ranvier, the latter is good for the arrangement of the neurokeratin. A convenient stain for the latter is dilute hematoxylin (1 part hematoxylin with 20 parts of distilled water) for some hours or overnight. This usually stains the sheath nuclei well.

To show especially the neurokeratin, the fresh nerves may be digested with trypsin or pepsin; or fixed in 95 per cent alcohol to dissolve the myelin, and stained with dilute hematoxylin and acid fuchsin. To make permanent mounts of isolated nerve-fibers, small pieces (not over 1 mm. in diameter) are stained, dehydrated in alcohols, cleared in clove oil, where they are carefully teased out, and mounted in balsam. In fixed material the connective tissue framework is tougher than in the fresh condition but the nerve fibers are likewise more resistant. The large myelinated fibers are easiest to isolate; the unmyelinated fibers require more careful teasing.

*Osmic Acid Method.* Osmic acid is the classical substance for demonstrating myelinated nerve-fibers, and is still one of the best. The myelin is blackened by the osmic acid, and between the blackened fibers are also to be seen many thin unmyelinated fibers. These are grayish in appearance, nearly colorless. Osmic acid is the method of election in the study of the size of myelinated fibers in relation to the rate of transmission of nerve impulses.

The osmic acid is used in  $\frac{1}{2}$  of 1 per cent solution, or at half this strength. As osmic acid dissolves slowly, the stock solution of 2 per cent or 1 per cent should be prepared beforehand.

In preparing the stock solution wash and dry the outside of the sealed glass tube in which the osmic acid crystals are bought. File around the middle of the tube and holding it within the folds of a clean towel, break it into two parts. Drop the pieces of the glass containing the osmic crystals into the appropriate quantity of distilled water in a glass stoppered bottle. If there is no contamination by organic substances, and if the solution is kept in a dark place, not exposed to high temperatures, it remains unreduced for several months. After some time, it becomes brownish in color, a black precipitate settles on the bottom of the bottle, and the solution is no longer useful.

In preparing material by this method it is well to use thin nerves, 1 to 2 mm. in diameter, as the osmic acid does not penetrate far. In the osmic acid it soon begins to darken but fixation should be continued twenty-four hours. Immersion in the osmic acid may be prolonged to a week but the nerve becomes brittle. The material is then washed in running water for twelve to twenty-four hours (small pieces one-half hour).

When the nerve-fibers are to be examined whole they are placed, after washing, in glycerin and are ready to be torn apart. If mounted in glycerin and the coverslip cemented around the edge, they remain unaltered for years. Or, after washing, small bundles of fibers may be dehydrated by passing them directly into 95 per cent alcohol (changed twice, two to three minutes in each) and then cleared in clove oil for three to five minutes. They may be further torn apart, and then mounted in balsam under a cover glass.

In a study of the length of internodes, Takahashi<sup>1</sup> used the following procedure in isolating the fibers of the sciatic nerve of frogs:

A short piece of the fresh nerve, on a strip of cardboard, was fixed and at the same time macerated by leaving it twenty-four hours in the following solution:

<i>Solution A</i> Osmic acid, 1 per cent.....	5 parts
Chromic acid, 0.25 per cent.....	3 parts
Hydrochloric acid 0.10 per cent.....	2 parts

Wash in running water twenty-four hours.

Transfer to solution B twenty-four hours.

<i>Solution B</i> Glycerin .....	10 parts
50 per cent alcohol.....	20 parts
Hydrochloric acid.....	0.09 parts

After this the specimens are preserved in solution C.

<i>Solution C</i> Glycerin.....	10 parts
50 per cent alcohol.....	20 parts

This is renewed once or twice at intervals of twenty-four hours.

### III. Methods of Preservation

1. Formol, 10 per cent; Bouin's Fluid; Orth's (formol-Müller) Fluid; Formol and Alcohol Mixtures. Human brains are generally preserved entire in 10 per cent formol (i.e. 10 parts of the commercial product with 90 parts of water) and may be kept in it for years without change in outer form, but with a certain increase in size and some loss of solids. Formol solutions penetrate rapidly and give the tissue a firm

<sup>1</sup> Takahashi, K. *J. Comp. Neurol.*, 18: 167, 1908.

consistency. After formol fixation the material may be imbedded in paraffin or collodion and the sections stained by routine methods.

For brains of fetuses and the very young it is well to use 10 per cent formol made up with a half-saturated solution of alum in water, in order to increase the hardening.

By adding to 5 per cent formol solution enough sodium chloride to make the specific gravity 1.030 (about that of the human brain) the initial change is much less and there is usually a decrease in weight. As the brains float, submerged in the mixture, there is no distortion due to pressure on the sides of the container, and this is highly desirable in preserving human brains, whether intended for dissection or for microscopic study.

Formol also serves as the fixing agent in several special procedures: as the myelin stain method of Weigert, the silver impregnation method of Bielschowsky, and some of the modifications of the reduced silver method of Cajal.

Other fixing fluids suitable for preserving whole brains or large portions of them, are Bouin's, Orth's, and Müller's fluids. They are used as for other tissues, and histological sections from material thus preserved are stainable by all the routine methods.

#### IV. Macroscopic Staining of Gray and White Substance

Several methods have been developed in recent years for showing striking color differences between gray and white substance in slices of brain hardened in formol. These preparations are intended mainly for naked-eye examination. Sincke<sup>2</sup> placed the slices first in an aqueous solution of iron chloride, and then after washing, in an aqueous solution of potassium ferrocyanide. The gray matter was stained a dark blue and the white matter a very pale blue. However, fading occurred in a comparatively short time.

Mulligan<sup>3</sup> immersed the slices in a fat solvent for a short time, prior to the staining process. This was for the purpose of making a protective film over the white matter, so that it would not take up the stain. The idea was that the lipoids of the myelin would be softened and be spread over the intervening axones and neuroglia of the white substance, while the gray substance would remain exposed to the action of the stain. The solution found by Mulligan to be best adapted for this purpose was a phenol mixture made up as follows:

<sup>2</sup> Sincke, G. *Anat. Anz.*, 61: 311, 1926.

<sup>3</sup> Mulligan, J. H. *J. Anat.*, 65: 468, 1931.

Carbolic acid, crystalline.....	40	gm.
Copper sulphate, crystalline.....	5	gm.
Hydrochloric acid, conc. ....	1.25	c.c.
Water.....	1000	c.c.

In staining he used tannic acid solution followed by iron alum.

Recently LeMasurier<sup>4</sup> has selected certain parts from these two methods and has combined them into a procedure by which the contrast between gray and white matter is sharp, with the coloring brilliant and lasting.

After formol fixation the slices are washed for twelve to twenty-four hours in running water and in distilled water for one hour with three changes. One or two of the slices are then placed for two minutes in an ample amount (e.g. 500 c.c.) of Mulligan's solution of phenol at 60°-65°C. They are then washed in a large volume of cold tap water one minute and placed in a 1 per cent solution of ferric chloride in distilled water for two minutes. They are now washed in running water for five minutes, and placed in a 1 per cent solution of potassium ferrocyanide in distilled water until the gray matter is a brilliant blue. This should take not longer than three minutes. All the slices from one brain may be stained without renewing the solutions. Wash in running water for twenty-four hours and finally preserve in 70 per cent alcohol or 10 per cent formol.

## V. General Histologic Structure

In the study of growth of layers, as of the cerebral cortex, or of cells, as of Purkinje cells, it is necessary to select as fixing fluid one which produces as little deviation as possible from the size in the living condition. Bouin's fluid has been largely used for this purpose on mammalian tissues in recent years.

Several factors, in addition to the fixing fluid itself, have a considerable influence on the final size of the brain. These are freshness of the brain, presence or absence of soft membranes, age, initial size of brain, amount of fluid used and temperature.

Plant<sup>5</sup> studying the factors influencing the behavior of the brain of the albino rat in Müller's fluid, found a rapid swelling for a week and then a steady loss until the seventy-fifth-day weighing, when the brain still weighed 20 to 30 per cent more than the fresh weight. The factors to which this range in amount of swelling (20 to 30 per cent) was due were chiefly: age of animal, initial brain size, and percentage of water

<sup>4</sup> LeMasurier, H. E. *Arch. Neur. Psychol.*, 34: 1065, 1935.

<sup>5</sup> Plant, J. S. J. *Comp. Neurol.*, 30: 411, 1919.

in the brain. It is evident that fallacious results might follow from the use of this fixing fluid in growth studies.

Hrdlicka,<sup>6</sup> using both human and animal brains, studied the effects of formol solutions, and found a sharp initial rise in the weight of the specimens, reaching a maximum within less than a week, with a subsequent gradual, long-continued loss. On sheep brains 10 per cent formol caused an increase in weight of 15 per cent by the end of the first week, which was reduced to 10 per cent at the end of the second month. This gradual loss is attributed to the dissolving of certain brain constituents by the watery fluid.

H. D. King<sup>7</sup> studying the effects of 10 per cent formol on brains of albino rats, found that it produced a pronounced swelling at all ages, and that a 10 per cent solution of formol, neutralized with sodium carbonate, produced a much greater swelling than does a solution with a faintly acid reaction. Also 10 per cent formol, acting for periods of one month and over, extracts solids from brains of all ages. Brains of very young rats (from birth to ten days) lose 30 per cent of their solids, while brains of adults lose 2 per cent.

By histological examination of brains fixed in 10 per cent formol, King<sup>8</sup> showed that this substance did not have as injurious an effect on the structure of the cells as did other fixatives that produced much less alteration in brain weight. After fixation in 10 per cent formol and imbedding in collodion, there was no apparent shrinkage of the cell-body, the cytoplasm stained evenly and appeared uniformly distributed. The nucleus, however, was decidedly larger than normal, its reticulum poorly preserved and faintly staining. Of the many combinations of formaldehyde which King tried, the picro-formol-acetic mixture of Bouin was the one which produced practically no alteration in brain weight, and besides gave an excellent preservation of the nerve-cells.

Sugita,<sup>9</sup> in preparation for an intensive study of the growth of the cerebral cortex and of the pyramidal cells in the albino rat, compared the effects on brain weight and histological structure of the following fixatives: (1) Bouin's fluid, (2) 10 per cent formol, (3) 95 per cent alcohol, (4) Müller's and Orth's fluids, (5) Ohlmacher's modification of Carnoy. Specimens were imbedded in both paraffin and collodion for comparison. He found the Bouin's fluid best adapted for his purposes, inasmuch as there was no significant change in total weight or volume, and the original shape was quite well preserved though a slight shrink-

<sup>6</sup> Hrdlicka, A. *Proc. U. S. Nat. Mus.*, 30: 245, 1906.

<sup>7</sup> King, H. D. *J. Comp. Neurol.*, 23: 283, 1913.

<sup>8</sup> King, H. D. *Anat. Record*, 4: 213, 1910.

<sup>9</sup> Sugita, N. *J. Comp. Neurol.*, 28: 511, 1917; 29: 119, 1918.

age occurred, no matter what the age of the brain. The contours of the nuclei were rounded, and the Nissl substance showed well after staining. For each brain he used 20 c.c. of Bouin's fluid, and fixed for twenty-four hours at room temperature (or two hours at 37°C.); washed in running water, twenty minutes; into 20 c.c. 80 per cent alcohol, twenty-four hours; into 90 per cent alcohol, twenty-four hours; sliced pieces 2 mm. thick, and placed in absolute alcohol, six hours; xylol, one and one-half hours at room temperature; xylol-paraffin, one and one-half hours, at 37°C.; paraffin, two hours, in oven at 56°C.; stained with carbol-thionin (p. 450), washed, dehydrated, cleared and mounted.

Every step in the preparation of nerve tissue after fixation still further alters its dimensions. The dehydrating fluids cause shrinkage as does also the imbedding process, especially in paraffin. The aqueous solutions through which the tissues are passed tend to increase the volume, while sectioning and affixing to the slip, especially when the temperature is high, cause shrinkage, as do some of the dyes used for staining, and this shrinkage is increased by the dehydration preceding mounting.

By making linear measurements at definite points in the fresh material and at the same points on the imbedded specimen, and by comparing these with the measurements made on the section after mounting, an estimate as to the changes in size can be obtained.

All size observations should be reduced to those for the fresh material, for only in this way can the results obtained by different workers, and from different animals, be properly compared.

## VI. Shape and Relationship of Neurons

1. **Golgi Silver Methods.** These consist essentially of immersing fresh pieces of nervous tissue first in a solution containing potassium dichromate (and usually osmic acid), and then in silver nitrate. A black deposit of a reduced silver salt is formed in and around the processes and cell-bodies of many of the neurons. Only a small fraction of the neurons and neuroglia are thus brought into view, one here and one there, but these are often shown in their entirety, so that the form of their cell-bodies and the distribution of their processes stand out prominently on a nearly colorless background. This selectiveness is a great advantage, for if all neurons were equally blackened the individual would be masked. In addition to the neurons, however, other structures, as blood vessels and connective tissue fibers may be blackened, and there is often an irregular distribution of black precipitate, especially in the outer portion of the block. As the method is largely empirical, results

are uncertain, but usually something of interest is to be seen. With Golgi's original methods the best results are obtained with neonatal animals up to two to three weeks old.

There are three main methods of Golgi himself, of which only the rapid method is given here.

a. *The Rapid Method of Golgi.*

1. Fix and harden a number of thin pieces of brain or spinal cord, preferably not over 5 mm. thick, in the following solution (Golgi's fluid) for two to eight days:

Potassium dichromate, 3 per cent to 5 per cent aqueous . . .	4 parts
Osmic acid, 1 per cent aqueous . . . . .	1 part

This should be renewed if it becomes turbid.

2. Take out a piece beginning with the second day, and each successive day, rinse in distilled water and dry the outside with filter paper.

3. Dip in small amounts of 1 per cent aqueous solution of silver nitrate until precipitate no longer appears, then place in ample fluid and leave for one to two days. The material may be kept at room temperature or on an oven, not over 35°C., and it is usual but apparently not necessary to exclude the light.

4. Rinse in 80 per cent alcohol, several changes, to wash out the silver nitrate.

5. Thick sections are cut free-hand, or as frozen sections, or after rapid imbedding in collodion or paraffin.

The best results are obtained after *rapid imbedding in collodion*, as follows:

Transfer to 95 per cent alcohol for one to four hours according to size; absolute alcohol, for the same length of time; absolute alcohol and ether, one hour; thin collodion, in a loosely corked vial on the oven for several hours, until the collodion thickens; block; harden in vapor of chloroform; cut thick sections in 95 per cent alcohol.

The sections may be cleared in cedar oil, or creosote, followed by xylol, or in a mixture of carbolic acid 50 c.c., oil of thyme or cedar wood 50 c.c., oil of bergamot 25 c.c. followed by xylol. The sections may be cut in this mixture rather than in alcohol, so that the clearing process starts immediately. If the blocks of imbedded tissues are not cut at once they should be stored in this mixture.

There are two methods of mounting the sections:

a. Cover the sections on the slip with a relatively large quantity of thick xylol balsam, and heat carefully over a flame or on a hot plate to drive off the solvent, so that the balsam becomes hard as soon as it cools. Just before the balsam cools, a warm cover glass may be placed on the preparation and gently pressed down.

b. Melt some hard balsam in a porcelain crucible. Warm a slip, so that it is not too hot to hold, place the sections on it, and cover with a drop of the fluid balsam. It hardens immediately, as it cools. A cover glass may be added as before.

The duration of the hardening process in the Golgi's fluid is of great importance. This varies with the kind of impregnation aimed at, the region of the nervous system, age of the individual, whether embryonic or adult, species, temperature at which the hardening takes place, and quantity of hardening fluid. The times for the spinal cord are given as two to three days to show neuroglia, three to five days for nerve-cells, five to seven days for nerve-fibers and collaterals, but these are true only in a very general way, for neuroglia and nerve-fibers usually appear irrespective of the duration in the hardening fluid.

When the quantity of the tissues is greater the fluid is relatively less effective, and the hardening process is slowed. When there is under-hardening there appears only a diffuse red precipitate of silver chromate. When there is over-hardening there is absence of impregnation, and only sharply defined crystals. As to age, generally the older the tissue, the longer the time required in the hardening fluid. Fetal and newborn human tissues, under favorable conditions, react for twenty-four hours after death.

*b. The double impregnation process of Cajal.*

A sample procedure with pieces of cerebellum of a fifteen-day or thirty-day-old cat or dog is as follows:

Five or six flat pieces, not over 4mm. thick are placed in 30 c.c. of Golgi's osmic-dichromate fluid for three days on the oven at 20° to 25°c., in a glass-stoppered bottle. A stock of this fluid may be kept in a brown bottle in the dark. Place the pieces directly in a small quantity of 0.75 per cent silver nitrate, where there is a copious precipitate; then into 100 c.c. of 0.75 per cent silver nitrate, for thirty hours, on the table. Dry the outside of the pieces by turning them over on filter paper, and place them in the original 30 c.c. of osmic-dichromate fluid, and leave for one day on the oven. Change the pieces directly into the original 100 c.c. of 0.75 per cent silver nitrate, and leave for one day.

After rinsing off the silver in water, dry the outside of the pieces on filter paper. Dip them into 95 per cent alcohol for one to two minutes, and dry on filter paper. Mount directly on cubes of hard paraffin, by heating one surface of the latter with a heated spatula until the paraffin begins to melt. Place a piece of tissue on the melted surface, and apply a hot needle to paraffin around the base of the piece of tissue, to ensure good contact. Dip in 95 per cent alcohol. Cut sections 85 to 100 $\mu$ . Wash sections well in 95 per cent alcohol using 5 to 6 changes, total time not more than a half hour. Transfer sections to slip, press gently and dry with filter paper. In the Instituto Cajal the original formula of Cox<sup>10</sup> is still used for covering the Golgi and Cox-Golgi preparations, without the use of cover glasses. This consists of sandarac 75 gm., camphor 15 gm., turpentine 30 c.c., lavender oil 22.5 c.c., absolute alcohol 75 c.c., castor oil 5-10 drops. After the sections are placed on the slip, the 95 per cent alcohol is

<sup>10</sup> Cox, W. H. *Arch. f. Mikr. Anat.*, 37: 16, 1891.



blotted off carefully and the sandarac mixture applied directly. When firm, repeat application once or twice, until there is a permanently smooth surface, and protect from dust and light. Such preparations have been kept for twenty years and more without deterioration of the impregnation.

*c. Modifications of Golgi Methods with Formol.* These modifications react better with adult than with young tissues, and may be used on human autopsy material.

(1) Strong's formula<sup>11</sup> for the hardening fluid:

Potassium dichromate, 3½ to 5 per cent. ....	100 c.c.
Formol, full strength. ....	2½ to 5 c.c.

Use a number of thin flat pieces, and each day, for five to eight days, transfer a piece to 1 per cent silver nitrate, and proceed as in the original method. As the formol penetrates better than the osmic acid, the stage of the hardening process which is favorable for impregnation may be reached as early as eighteen hours. It is found, also, that this favorable stage lasts longer than with the osmic acid mixture, and consequently good results are more certain over a greater range of time.

(2) In Kopsch's modification,<sup>12</sup> the tissues are first placed in a mixture of 3.5 per cent potassium dichromate, 4 parts, and formol, 1 part, for twenty-four hours, and then in 3.5 per cent potassium dichromate alone for three to six days, before being silvered. The Purkinje cell dendrites and other structures of the cerebellar cortex are often well shown in human autopsy material by this method.

**2. Cox-Golgi Method.** This is one of the simplest procedures for demonstrating the relations of dendrites and axons to the cell-body.

There are three stock solutions which keep indefinitely.

- A. 5 per cent aqueous solution of potassium chromate.
  - B. 5 per cent aqueous solution of potassium dichromate.
  - C. 5 per cent aqueous solution of mercuric chloride.
- Dilute 16 c.c. of A with 40 c.c. of distilled water.  
 To 20 c.c. of B add 20 c.c. of C.  
 Mix these two solutions and allow to warm on incubator.

This quantity is suitable for the brain of an albino rat. To prevent evaporation, seal around the stopper of the bottle with paraffin, and leave on incubator for about a month. At the end of several weeks the tissue becomes brownish in color and rather friable. It may be tested by slicing off portions with a safety razor blade or making frozen sections. To obtain good sections, it is best to imbed in collodion in the usual

<sup>11</sup> Strong, O. *S. Anat. Anz.*, 10: 494, 1895.

<sup>12</sup> Kopsch, F. *Anat. Anz.*, 11: 727, 1896.

way. As the sections are to be cut thick, they may be imbedded rapidly. The sections should be well dehydrated and cleared before mounting in damar or balsam. They may also be mounted like other Golgi preparations in hard balsam or in the sandarac mixture of Cox, without cover glasses.

The cell-bodies and processes are outlined in black on a light yellowish or colorless background. There are also irregular black deposits and some blood vessels may be impregnated, but in both these respects the Cox method is often better than the Golgi silver methods. Preparations mounted under cover glasses usually deteriorate in several years.

Bremer<sup>13</sup> found that after thorough imbedding (usual steps of dehydration, followed by one week or more in thin collodion, and an equal time in thick collodion) the sections may be easily stained with hematoxylin and eosin, without injuring the impregnation, and that these stained specimens showed less of the mercury deposit after several months than did the specimens without stain.

3. The **Cajal reduced silver methods** show the size and relationship of neurons especially in embryos and neonatal animals. These methods are given later.

## VII. Methods for Special Structures in Cell-Bodies

1. **Nissl Bodies.** Nissl bodies have been demonstrated in the cytomes of nerve cells of animals as soon as twenty-five seconds after death by decapitation (Heldt<sup>14</sup>). They also persist for some time after death. Malone<sup>15</sup> for his study of the superior olive, used an entire human medulla, removed from the cranium seventeen hours post-mortem. From its living semi-fluid, diffuse state the Nissl substance changes into the form of granules, which have more or less characteristic shapes and patterns of arrangement in different types of nerve cells.

### *a. Simple methods for Nissl bodies.*

(1) Fix tissue, just removed from body, in 95 per cent alcohol, or 10 per cent formol, for one to two days.

(2) Imbed in paraffin, cut and mount sections, decerate and carry back to water.

(3) Stain for six hours to overnight in a 0.1 to 1 per cent aqueous solution of toluidine blue, methylene blue or cresyl violet.

(4) Rinse in water, dehydrate and differentiate in 80 per cent, 95 per cent and absolute alcohol. Clear in xylol and mount in damar or balsam.

<sup>13</sup> Bremer, J. L. *Anat. Record*, 4: 263, 1910.

<sup>14</sup> Heldt, T. J. *J. Comp. Neurol.*, 23: 315, 1913.

<sup>15</sup> Malone, E. F. *J. Comp. Neurol.*, 35: 205, 1923.

King's<sup>16</sup> carbol-thionin staining method has been used by a number of investigators at The Wistar Institute on the nervous tissue of the albino rat. The mounted paraffin sections or loose collodion sections are stained for two to three minutes in a 1 per cent solution of carbolic acid saturated with thionin. The sections are then washed quickly in distilled water, differentiated in 95 per cent alcohol, and passed quickly through a mixture of equal parts of chloroform and absolute alcohol into xylol and mounted in balsam. The mixture of chloroform and absolute alcohol must be changed frequently, especially on humid days.

Neutral red in dilute solution, well ripened, was found by Johnston<sup>17</sup> to be especially useful after formol fixation. The neutral red must be ripened, in 1 per cent aqueous solution, for one, two or four years. For use it is diluted to  $\frac{1}{4}$  or  $\frac{1}{10}$  of 1 per cent, and this diluted stain may be used repeatedly. It is advised to clear the sections in 1 part xylol with 2 to 3 parts of castor oil. Good results were obtained in sections  $50\mu$  thick, through the whole brain of the newborn child. It may also be used after the Cajal or Bielschowsky methods, on alcohol- or formol-fixed material.

Kirkman<sup>18</sup> was able to use an unripened neutral red solution on Bouin or formol fixed material with satisfactory results. The formula was as follows:

Neutral red (Coleman and Bell).....	1 gm.
Distilled water.....	500 c.c.
1 per cent aqueous solution of glacial acetic acid.....	2 c.c.

This was also used after Pal-Weigert stain. The sections were counter-stained for ten to twenty minutes, rinsed quickly in distilled water, differentiated in 95 per cent alcohol, dehydrated, cleared and mounted.

Huber's<sup>19</sup> technique has been used successfully on a large variety of vertebrate forms. Larger pieces than usual can be fixed, and the fixative penetrates rapidly enough for use on human autopsy material.

Fixing solution:

Ethyl alcohol, 95 per cent.....	100 c.c.
Acid trichloroacetic (Mallinckrodt).....	1.5 gm.
Mercuric chloride, c.p. (Mallinckrodt).....	3.0 gm.

Pieces 2 cm. thick are fixed two to three days, 4 cm. thick three to four days, larger pieces eight to ten days, with the fluid renewed every

<sup>16</sup> King, H. D. *Loc. cit.*

<sup>17</sup> Johnston, J. B. *Anat. Record*, 11: 207, 1916.

<sup>18</sup> Kirkman, I. J. *Anat. Rec.* 51: 323, 1932.

<sup>19</sup> Huber, G. C. Contributions to Med. Sc. Dedicated to Aldred Scott Warthin. Ann Arbor, Mich., 1927, 1.

two days. The fluid is decanted and replaced with 95 per cent alcohol, in which the tissues may be stored until further use. The mercuric chloride is not removed by iodinated alcohol, as this interferes with the staining. Dehydrate blocks thoroughly, imbed in paraffin, cut sections, remove paraffin, and carry down to water. Stain in 0.1 per cent aqueous solution of toluidin blue (Grübler) for fifteen to eighteen hours.

The stain is prepared by placing 1 gm. of toluidin blue in a liter flask and adding about 500 c.c. of distilled water. Heat gently and when the stain is dissolved, add 500 c.c. more of distilled water.

Decant staining solution, and wash the sections several times in distilled water. Decant distilled water, and fill the staining dish with a solution of lithium carbonate prepared as follows:

A saturated solution of lithium carbonate is made up by adding about 5 gm. of lithium carbonate to 1 liter of distilled water. Boil several minutes, cool and filter. To 100 c.c. of the saturated solution add 900 c.c. of distilled water.

The sections stay here for two hours, are washed for a few minutes in distilled water, and differentiated in 70 per cent alcohol, five to thirty minutes. The slides should now be placed flat in 95 per cent alcohol, for five to fifteen minutes, then in absolute alcohol, xylol. Mount in balsam.

Einarson stained the nerve cells of different parts of the nervous system of rabbit after various fixations (96 per cent alcohol, neutral formol, formol-alcohol, Zenker, Zenker-acetic, mercuric chloride, pure acids, ammoniated alcohol, alkaline-alcohols, pyridin, osmic acid, salts of different metals and other fixatives). He found by application of new staining methods that the Nissl pattern was just minimally modified by varying the fixative. Only the capacity of the cell for being stained with ordinary dyes was modified and changed by the selection of fixative. By the use of gallocyanin and gallamin blue, on alternate slides, he found that the Nissl substance was practically always stainable. He suggests that the Nissl bodies contain at least three histological components, i.e., (1) a basophil chromatin substance, (2) a basophil protein, and (3) an acidophil protein. The first two are stainable with gallocyanin and the third with gallamin blue and slightly with eosin. After any ordinary fixation all of the histological components can be stained by alternating the use of gallocyanin and the gallamin blue methods.

#### Galloycyanin Method<sup>20</sup>:

One gram of chromalum ( $K_2SO_4 \cdot Cr_2SO_4 \cdot 24 H_2O$ ) is dissolved in 200 c.c. of distilled water to which is added 0.3 gm. of gallocyanin (Grübler-Hollborn). The mixture is then gradually warmed and gently boiled for fifteen to twenty-five

<sup>20</sup> Einarson, L. *Am. J. Path.*, 8: 295, 1932.

minutes, shaking frequently. Cool gradually and filter. Stain the deparaffinized sections after they have been carried down through water, twenty-four to forty-eight hours. Wash in distilled water, dehydrate, clear and mount.

#### Gallamin Blue Method<sup>21</sup>:

Five-tenths gram of gallamin (Grübler-Hollborn) is gradually heated in 200 c.c. of distilled water, and allowed to boil gently for five to ten minutes. Cool slowly and filter. Stain sections overnight twelve to twenty-four hours. Wash in water three to eight minutes, differentiate in 50 per cent alcohol fifteen seconds to three minutes, 70, 80, 95 per cent and absolute alcohol for three minutes each, clear and mount. The stain comes out readily in 70 per cent alcohol and less in the higher alcohols.

When the processes of nerve cells are injured there are chemical and morphological changes in the Nissl substance. When the cell does not degenerate completely as the result of the injury, the Nissl substance gradually reforms into the normal Nissl bodies. In general, the same methods are used for showing degenerative and regenerative changes as for the normal Nissl bodies. Nicholson<sup>22</sup> has found in the albino rat, after ligation of the axons of the hypoglossal nerve, that degeneration progressed from the first to the fifteenth day, and that regenerative processes took place and steadily progressed from the sixteenth to the forty-fourth day. Papez,<sup>23</sup> studying the subdivisions of the facial nucleus by the method of chromatolysis, removed the tissues to be examined from rat, guinea pig, cat and dog, at the thirteenth to the seventeenth day after operation. Windle,<sup>24</sup> after removal of the pulp from the teeth of dogs, found chromatolytic changes in certain cells of the Gasserian ganglion after twelve to fourteen days. Both Papez and Windle fixed in Carnoy's fluid; the former stained with methylene blue, the latter with toluidine blue.

**2. Neurofibrils.** *a. Cajal's Reduced Silver Methods.* The central idea of Cajal's methods is the application of photographic developers to tissues which have been treated with silver nitrate. In all the methods except the original there is a preliminary fixation before the immersion in silver nitrate. The neurofibrils are seen within the neurons, and the axons, dendrites, and telodendria are impregnated with a reduced silver compound.

Many modifications have been introduced into the original method to adapt it for special purposes. These have been published in a special

<sup>21</sup> *J. Comp. Neurol.*, 61: 101, 1935.

<sup>22</sup> Nicholson, F. M. *J. Comp. Neurol.*, 36: 37, 1923.

<sup>23</sup> Papez, J. W. *J. Comp. Neurol.*, 53: 159, 1927.

<sup>24</sup> Windle, W. F. *J. Comp. Neurol.*, 43: 347, 1927.

book<sup>25</sup> which may be referred to for a complete account. These modifications consist principally in the chemicals used in the preliminary fixation, and the presence or absence of a secondary fixation. The procedure consists of (A) primary fixation, (B) secondary fixation, (C) washing, (D) silvering, (E) washing, (F) reduction. After reduction the tissues are rinsed in distilled water, hardened in absolute alcohol, imbedded in collodion or paraffin, sectioned, cleared and mounted in balsam or damar. The following are the procedures in use at the Instituto Cajal, from personal observation in 1928, together with certain modifications suggested mainly by American investigators. All chemicals should be chemically pure, especially in the fluids used for the preliminary fixations. The alcohol for fixation, both when used alone and in the ammoniacal alcohol fixation, may be absolute or 96 per cent, but should be high-grade, as for analysis. Cajal used formaldehyde bought in one pound containers and pyridine in small ampules which were re-sealed after use. Experience has shown that the selection of the formula to be used depends on the part of the nervous system to be studied, on the age of the animal, and to a certain extent on the species. Pieces should be not over 3 mm. in thickness ordinarily.

*Spinal Cord.* All the formulas are applicable to the spinal cord. The neurofibrils of the motor neurons of the ventral horns are shown well by Method III, especially in young animals, during the first two weeks after birth. For myelinated fibers, large and small, Methods II and VI are effective; for the terminal buttons of Cajal, the myelinated plexus of the gray matter and the myelinated fibers of the white matter, Methods III, IV, and V.

*Cerebellum.* The cerebellum responds to the different formulas with a considerable variety of reactions. For the adult Purkinje cells, and the terminal pericellular arborizations of the basket cells, Method III; for adult mossy fibers, Methods IV and VI; for climbing fibers, parallel fibers, axons of stellate cells, Method VI (this method is especially good for these structures in small adult animals). The method of Cajal for use on frozen sections is excellent for the baskets around the Purkinje cells. For embryos, especially the younger stages, Method V. For the granule cells, Methods I and II, and sometimes III.

*Cerebrum.* In the cerebrum Method I is advantageous for the pyramidal cells, especially the medium and small cells, particularly in young animals, as cat and dog, eight to thirty days; for unmyelinated and myelinated fibers, of medium and larger size, Methods II and III; for fine nerve plexuses, Methods IV, V, and VI.

<sup>25</sup> Cajal, S. R., and deCastro, F. *Elementos de Técnica micrográfica del sistema nerviosa*. Madrid, 1933.

*Neuroblasts.* For the demonstration of neuroblasts and their processes, the best results are given with alcohol, and with pyridine. These are useful with all the vertebrates, especially with embryos of birds and of fish. For more advanced stages of embryos and fetuses of mammals, however, Methods III and VI and alcohol with accelerator may be used.

*Ganglia.* For sympathetic ganglia are advised Methods III, IV and V. The visceral ganglia (plexuses of Auerbach and Meissner, etc.) are more difficult to color. For these Castro has obtained good results with formulas containing chloral hydrate and urethane. For sensory ganglia Methods II, III and V are used.

**Method I** is applicable to the brains of small animals or to those of fetuses and the very young of large animals. It gives good results with the medulla, pons, cerebellum, cerebrum of fetal and neonatal animals, and with the cerebellum and cerebrum of adults. It shows pyramidal cells in the cerebrum and granule cells in the cerebellum.

Without any previous fixation the pieces of tissue are:

(D) Silvered in a 1.5 per cent aqueous solution of silver nitrate for three to five days, at 37°C. in the dark. The silver solution should be abundant, 80 to 100 c.c. for 2 or 3 pieces of tissue, and should be kept in glass bottles, with ground stoppers or with corks paraffined to prevent evaporation. The impregnation is sufficient when the pieces have assumed the color of tobacco.

(E) Rinse the tissues in distilled water.

(F) Reduce for twenty-four hours in the following:

Pyrogalllic acid or hydroquinone.....	1 to 2 gm.
Neutral formalin, full strength.....	5 to 10 c.c.
Distilled water .....	100 c.c.

In invertebrates, especially with *Hirudo*, good results have been obtained using 6 per cent silver nitrate.

Before mounting the sections, it often improves the clearness of the preparations to tone them in a bath of gold chloride, as follows:

Water.

Yellow chloride of gold 1 to 500 parts of water for fifteen to thirty minutes.

Water.

Hyposulphite of soda 5 or 10 per cent for one-half minute.

Water.

Dehydrate etc. and mount in balsam.

**Method II** colors splendidly both myelinated and unmyelinated nerve-fibers, pericellular arborizations and large and medium-sized nerve cell-bodies. Best results are with cerebellum and cerebrum. Impregnates very well also the motor and sensory nerve-terminations (corpuscles of Pa-

cini, Meissner, Krause, Merkel, etc.). Gives good results also with nerves in course of regeneration, with young embryos (embryos of chicks from sixty hours onward) and with young fish.

(A) Fix in alcohol, absolute or 96 per cent for twenty-four hours. In place of ethyl alcohol one may use methyl, propyl, butyl, or allyl alcohol.

(B) Cut out pieces not over 2.5 mm. thick and silver for five to seven days, at 28° to 35°C.

(E) and (F) as before.

#### *Variant I.*

(A) To the alcoholic fixative is added a hypnotic (veronal, chloral hydrate, etc.) as an accelerator, 1 gm. to 50 c.c. of 96 per cent or absolute alcohol.

This requires only five days in the silver nitrate and is said to increase the constancy and regularity of the reaction. With very young embryos, however, the addition of the accelerators, e. g., veronal, does not give as good results as does the alcohol alone.

Material which has been for a long time in alcohol may be rejuvenated by chloral hydrate or veronal. Thus, pieces of human cerebrum and cerebellum which have been in 95 per cent alcohol for a whole year and which then give but a weak coloration, acquire a greater electivity after treatment with chloral hydrate or veronal. The same effect may also be obtained with ammoniated alcohol.

Method III is particularly good for the neurofibrils of spinal cord and the ganglia of neonatal dogs, cats and rabbits. Good results are obtained with the adult cerebrum and cerebellum and sympathetic system. For the last, especially in man and other large mammals, this formula is the best, according to Castro.<sup>26</sup>

(A) Fix for twenty-four hours in

Absolute or 96 per cent alcohol..... 50 c.c.

Ammonia, concentrated ..... 1 to 12 drops

The ammonia should be fresh from the stock bottle. It is important to modify the quantity of ammonia according to the part of the nervous system to be studied. For spinal cord and medulla, 8 to 12 drops; for cerebrum, 1 to 3 drops; for cerebellum 4 drops; for peripheral nerve-endings, 2 to 3 drops. If the quantity of ammonia is excessive, the coloration is pale. In order to insure uniform size of the drops one should use a very fine tube, as a 1 c.c. pipette, calibrated to hundredths of a cubic centimeter. In the fixation, excessive contraction may be avoided by first placing the pieces in 70 per cent alcohol for six hours, then in 85 per cent alcohol for an hour or two, and finally in ammoniated alcohol.

<sup>26</sup> de Castro, F. *Trab. Lab. Investig. Biolog. de la Univ. de Madrid*, 19: 241-340, 1921.



- (c) Mop with filter paper.
- (d) to (F) as before.

**Method IV.** Good results are obtained on the unmyelinated fibers of the central nervous system and the pericellular arborizations, especially of adult animals. It colors well the mossy fibers of the cerebellum.

- (A) Fix in 15 per cent formol for six to twelve hours.
- (B) Wash in running water, six hours or more, to remove formol completely, and refix in ammoniated alcohol (96 per cent, or absolute, alcohol 50 c.c. ammonia 5 drops) for twenty-four hours.
- (c) Mop with filter paper.
- (d) to (F) as before.

*Variant 1.* Walker<sup>27</sup> recommends for rat fetuses:

- (A) Fix in 10 per cent neutral formol, nine days.
- (c) Rinse in water.
- (D) Silver as in Method 1 for two days.
- (E) Rinse in water.
- (F) Reduce for twenty-four hours in the dark in:
 

Hydroquinone.....	2 gm.
Distilled water.....	100 c.c.
Neutral formol.....	8 c.c.

**Method V.** This gives good results especially in young embryos. In adults it colors by preference the fine myelinated fibers. The neurofibrils are strongly colored. It is excellent for nerve terminations and for the phenomena of regeneration. It, however, has the disadvantage of being somewhat inconstant, and of not revealing in the case of regeneration the earliest formations. But when properly treated the regenerating fibers are colored intensely. The intensity of color after treatment with silver nitrate is in general paler than that with the other formulas.

- (A) Fix for twenty-four hours in:
  - Pyridine undiluted, or in
  - Pyridine diluted with an equal quantity of distilled water, or in
  - Pyridine 40 parts and 95 per cent alcohol 30 parts.

It is best to use the diluted pyridine with adult tissues, to avoid undue distortion and vacuolization. Cajal employed usually the pyridine at 70 per cent and obtained good results in embryos on account of the great contrast and power of penetration.

- (c) Wash in running water, twelve to twenty-four hours, until the odor has gone, and leave in 95 per cent or absolute alcohol six to twelve hours.
- (d) to (F) as before.

<sup>27</sup> Walker, G. A. *J. Comp. Neurol.*, 59: 29, 1934.

*Variant I.* Faworsky<sup>28</sup> recommends for terminations in muscles and epithelia:

(A) Fix for twenty-four hours in 100 c.c. 50-80 per cent alcohol with 0.5-5 c.c. of acetic acid.

(B) Wash in 50 per cent alcohol several hours and refix in 96 per cent alcohol, 100 c.c., ammonia 1 c.c. for two days. Wash and place in pyridine for one to two days.

(C) Wash twelve to twenty-four hours in running water and in changes of distilled water two hours.

(D) Silver in 2 per cent solution 4 to 10 days at 37°C.

(E) Mop with filter paper.

(F) Reduce for twenty-four hours in:

Pyrogallol.....	1 gm.
Neutral formol.....	10 c.c.
Distilled water.....	100 c.c.

Dehydrate, starting with 70 per cent alcohol.

*Variant II.* Ranson,<sup>29</sup> using a combination of this method and Method III, has obtained particularly good results on unmyelinated fibers in the peripheral nervous system. It gives an excellent differential coloration of unmyelinated fibers, both in nerve-trunks, in ganglia and in pia mater. The unmyelinated fibers are black, and are sharply differentiated from the surrounding light yellow endoneurium; myelinated fibers are stained yellow, and are surrounded by a colorless ring of myelin. Ranson has used it for studying the myelinated and unmyelinated components of spinal nerves, and of the vagus nerve in several species, as well as spinal ganglia, sympathetic trunk and ganglia, and spinal cord. Other workers have used his modification with good results in the central nervous system.

(A) Fix in absolute alcohol with 1 per cent ammonia, forty-eight hours.

(B) Rinse in distilled water, less than three minutes and refix in pyridine, twenty-four hours.

(C) Wash in many changes of distilled water for twenty-four hours.

(D) Silver in 2 per cent solution in the dark at 35°C., three days.

(E) Rinse in distilled water.

(F) Reduce for twenty-four hours in:

Pyrogallol.....	4 gm.
5 per cent formol in distilled water.....	100 c.c.

Ranson and Billingsley<sup>30</sup> found that in dealing with small nerves and ganglia, the method failed to give good results, apparently because

<sup>28</sup> Faworsky, B. A. *Anat. Anz.*, 70: 376, 1930.

<sup>29</sup> Ranson, S. W. *Rev. Neurol. & Psychiat.*, 12: 467, 1914.

<sup>30</sup> Ranson, S. W., and Billingsley, P. R. *J. Comp. Neurol.*, 29: 313, 1918.

the volume of the tissue was too small. This was overcome by coating the nerve with other nervous tissue in the following manner: a fine silk thread was tied to the nerve, and by means of a long fine needle it was drawn into the lateral half of the spinal cord along the line of the ventral gray column. After fixation two hours in the ammoniated alcohol the block was pared down with a razor into the form of a bar, not over 4 sq. mm. in cross-area. The cord was dissected away from the nerve just before it was dehydrated and cleared in preparation for imbedding.

This method is also useful for showing the degeneration of unmyelinated fibers. Johnson,<sup>31</sup> in an experimental study of the degeneration of the extrinsic nerves of the small intestine by this method found the following procedure important in fixing the tissues: Physiological salt solution was injected through the mesenteric circulation and through the lumen of the selected length of the intestine until it was blanched. Then the ammoniated alcohol mixture was introduced by the same routes under slight pressure. The intestine was thus kept distended for an hour, when strips, 2 mm. or less in diameter, were cut off along the mesenteric attachment, threaded through pieces of spinal cord, and the whole returned to fresh fixing fluid to complete the fixation.

**Method VI** gives the best coloration for motor end-plates, pericellular arborizations, cerebellum, etc. The addition of the ammonia to the alcohol is necessary in order to render alkaline the acid fluid of the chloral hydrate, because the reaction takes place only in a slightly alkaline medium.

(A) Fix for twenty-four hours in:

Chloral hydrate .....	5 to 6 gm.
Absolute alcohol .....	25 c.c.
Distilled water .....	75 c.c.

(The original Cajal formula was chloral hydrate 5 gm., water 50 c.c., but Castro has suggested the addition of the alcohol, to restrain the swelling of the tissue.)

(B) Wash rapidly in water and refix in ammoniated alcohol (absolute alcohol 50 c.c., ammonia, 4 drops) for twenty-four hours.

(C)-(F) as before.

**Variant I.** In certain cases it is advantageous to increase the quantity of alcohol in the fixative. Good impregnation of terminations in muscles in crayfish was obtained by fixing in:

Chloral hydrate .....	5 gm.
Absolute alcohol .....	40 c.c.
Distilled water .....	40 c.c.

<sup>31</sup> Johnson, S. E. *J. Comp. Neurol.*, 38: 299, 1925.

*Variant II.* Another formula which gave good results with crayfish is:

Chloral hydrate.....	5 gm.
Pyridine.....	20 c.c.
Absolute alcohol.....	40 c.c.
Distilled water.....	40 c.c.

*Variant III.* Castro gives a formula with urethane. Both urethane and chloral hydrate color at one time the nerve-fibers and the nuclei.

Urethane.....	2 gm.
Absolute alcohol.....	40 c.c.
Distilled water.....	40-50 c.c.

*Variant IV.* For terminal buttons in the spinal cord of cat, Davenport<sup>32</sup> recommends:

(A) Perfusion with 10 per cent chloral hydrate and fixation of pieces for one to two hours in 10 per cent chloral hydrate.

(B) Pyridine 20-40 per cent for two days. He also uses this alone without previous fixation. If the blocks are left in chloral hydrate for twenty-four hours, ammoniated alcohol should be used as the refixer instead of pyridine.

(C) Wash for two hours in distilled water, changing half-hourly.

(D) Silver as before.

(E) Wash 30 minutes.

(F) Reduce in 20 per cent pyrogallol for five hours.

*b. Special Modifications of Cajal's Methods.*

(1) For Calcified Tissues. Cajal and Castro<sup>33</sup> have devised several formulas for combining decalcification with fixation.

*(a) Cajal:*

(A) Fix and decalcify, usually one to two days, in

Formol.....	14 c.c.
Distilled water.....	86 c.c.
Nitric acid.....	3 c.c.

(B) Wash carefully for one day and refix with ammoniated alcohol, or with a mixture of alcohol with pyridine (see Method v) for one day.

(C) If alcohol and pyridine are used be careful to wash well before passing into the silver nitrate. If ammoniated alcohol is used mop off.

(D) to (F) as before.

*(b) Castro.*

(A) Fix and decalcify in the following (until lime salts dissolve, one to two days):

Chloral hydrate.....	5 to 6 gm.
Absolute alcohol.....	25 to 40 c.c.

<sup>32</sup> Davenport, H. A. *Stain Technology*, 8: 143, 1933.

<sup>33</sup> de Castro, F. *Trav. Lab. Recher. Biolog. de la Univ. de Madrid*, 23: 427, 1925.

Distilled water..... 40 to 75 c.c.  
 Nitric acid ..... 3 c.c.

(B) Wash carefully, for twenty to twenty-four hours, and refix in ammoniated alcohol (4 to 5 drops of ammonia in 50 c.c. absolute alcohol) one day.

(c) to (F) as before.

(c) *Castro*.

(A) Fix and decalcify in:

Urethane..... 2 gm.  
 Absolute alcohol..... 40 to 60 c.c.  
 Distilled water..... 40 c.c.  
 Nitric acid ..... 3 c.c.

(B) Wash carefully.

(D) to (F) as before.

(2) For Fish Brains.

Bartelmez,<sup>34</sup> using *Salmo* and *Ameiurus*, fixed larval heads or brains of adults in acetic alcohol. For larval forms, a mixture of 10 parts of absolute alcohol with 1 part of glacial acetic acid gave the best results. For adults he fixed in

Absolute alcohol..... 19 parts	or	Absolute alcohol..... 19 parts
Glacial acetic acid..... 1 part		Glacial acetic acid..... 2 parts
		Chloroform..... 10 parts

Fixation for twenty minutes is usually sufficient; it should not be more than one and one-half hours; otherwise the material becomes too brittle. Rinse in 80 per cent alcohol, followed by distilled water, and then place in the silver nitrate solution, in the dark, at 35 to 40°C. The solution is changed each day, and the strength altered thus: first day 1 per cent silver nitrate; second day 1.5 per cent; third day 2 per cent; fourth day, 1 per cent again, and repeating as before, never going above 2 per cent silver nitrate. The total time in the silver baths varies from 3 to 8 days, usually best at four to five days, when the material becomes brown. Further treatment as in Cajal Method 1. This gives a preparation showing some cells in every nucleus stained with all their processes including the axon; and in many, the endings of the eighth nerve in the internal ear were well shown.

(3) For Large Blocks.

Gurdjian<sup>35</sup> has found the Cajal methods applicable to relatively large blocks. By the following modifications it was possible to impregnate successfully a whole brain stem of a seventeen-year-old boy, brain stem of a large adult dog, adult rat brains, pigeon brains, etc. To insure more rapid and uniform fixation the blood vessels of the part may be in-

<sup>34</sup> Bartelmez, G. W. *J. Comp. Neurol.*, 25: 87, 1915.

<sup>35</sup> Gurdjian, E. S. *J. Comp. Neurol.*, 43: 1, 1927.

jected with the fixing fluid, but this is not necessary with brains of albino rats or others of similar size.

(A) Fix in mixture of 100 parts of 95 per cent alcohol, with 1 part of concentrated ammonia for ten to twenty days (change daily). Mop before putting into a mixture of 100 parts of 95 per cent alcohol, and 2 to 5 parts of chloral hydrate. Leave for three to five days (changing daily). Rinse in distilled water.

(B) Place in ammoniated alcohol again, two to three days (two or three changes).

(c) Mop.

(D) Silver in 0.75 per cent solution, for two to four weeks in the dark, at about 30°C. (change twice a week).

(F) Reduce for a week to ten days, or longer if necessary in:

Pyrogalllic acid.....	5 gm.
Formol.....	5 c.c.
Distilled water.....	100 c.c.

(4) For Frozen Sections.

(a) By the use of a protective colloid in the reducing agent, Liesegang was able to adapt the Cajal method for frozen sections.

(A) Fix in 12 per cent formol and make frozen sections.

(D) Silver in 0.75 silver nitrate, for some hours until they become brown.

(F) Reduce for a few seconds in:

Hydroquinone, 0.5 per cent aqueous solution.....	1 part
Silver nitrate, 0.75 per cent aqueous solution.....	1 part
Gum arabic, 50 per cent aqueous solution.....	1 part

Wash, mount, dry with filter paper. Dehydrate in 95 per cent and absolute alcohol, clear in xylol and mount in balsam or damar.

All manipulations should be done with glass hooks.

(b) *Cajal's<sup>36</sup> modification.* By this method are colored intensely the arborizations of the basket cells around the Purkinje cells, and the mossy and climbing fibers of the cerebellar cortex, the calyces of Held in the nucleus of the trapezoidal body and the terminations in the ventral ganglion of the acoustic nerve.

(A) Blocks from large brains, or entire small brains are fixed in 20 per cent formol for three days or more. Pieces which have been in the fixative more than a year still give good results. Cut frozen sections at 30-40 $\mu$ . Wash for several minutes in distilled water.

(D) Place sections for four to six hours in the following mixture:

Silver nitrate, 2 per cent.....	12 c.c.
Pyridine, pure (e.g. Kahlbaum).....	7-10 drops
Alcohol 97 per cent.....	5-6 c.c.

The sections become light brown in color. This reaction takes place at the room temperature, but is more rapid and certain in the oven.

<sup>36</sup> Cajal, S. Ramón y. *Trav. Lab. Recher. Biol. Univ. de Madrid*, 24: 217, 1926.

(E) Rapid passage, two to four seconds' duration, through absolute alcohol, 8 to 10 c.c., in a glass or porcelain dish. In order to avoid excessive extraction of the nitrate, only 2 or 3 sections should be passed through at one time.

(F) Reduce one to three minutes in the following mixture, which may be kept in a stock bottle.

Hydroquinone.....	0.30 gm.
Distilled water .....	70 c.c.
Formol.....	20 c.c.
Acetone.....	15 c.c.

Wash in a large quantity of water.

It is best to tone the sections in gold chloride, as in Method 1.

(c) *O. Schültze's method.* P. Stöhr, Jr.,<sup>37</sup> used the following method in his study on the sympathetic system. Slight variations in strengths of solutions are used for other regions.

(A) Fixation in 10 per cent formol, not longer than six months. Cut frozen sections 30–40 $\mu$ .

(B) Refix for twenty-four hours in:

Sodium hydroxide .....	0.8 gm.
Distilled water.....	100 c.c.

(c) Wash two hours.

(d) Silver for twelve to twenty-four hours in a 10 per cent solution.

(F) Reduce a few seconds to one minute in:

Hydroquinone.....	2.5 gm.
Distilled water .....	100 c.c.
Formol.....	5 c.c.

Wash in two changes of distilled water, 96 per cent alcohol, clear and mount.

(5) For Sections Mounted on the Slide. This is useful when more than one technique is desired from one block of tissue.

(a) *Davenport*<sup>38</sup> uses the following procedure:

(A) Fix in formol. Blocks are imbedded in either paraffin or collodion. Paraffin sections are passed through xylol, absolute alcohol, alcohol-ether and 2 per cent collodion. The collodion sections are mounted on slides with egg-albumen and plunged into a 2 per cent solution of collodion. When the edges of the original collodion sections soften, the slide is removed, drained, inverted and tilted back and forth so that the coating will form evenly. It is then allowed to rest face up until the coating hardens. The slide is then placed in 80 per cent alcohol for a few minutes.

(b) Silver for one to four days at 37° to 40°C. in 10 gm. of silver nitrate dissolved in 10 c.c. of distilled water to which are added 90 c.c. of 95 per cent alcohol and 0.5 c.c. of 7 per cent nitric acid.

Some tissues require more acid. If too little acid is used the differentiation is

<sup>37</sup> Stöhr, P., Jr. *Mikr. Anat. des Veget. Nervensystems*. Berlin, 1928.

<sup>38</sup> Davenport, H. A. *Anat. Rec.*, 44: 79, 1929.

not good. The time in this bath can be judged by the appearance of a light tint in the tissue. Direct sunlight or long exposure to direct daylight should be avoided.

(E) Wash in 95 per cent alcohol.

(F) Reduce in:

Pyrogallol.....	5 gm.
Neutral formol.....	5 c.c.
Alcohol, 95 per cent.....	100 c.c.

To keep down the formation of precipitates in the reducer a few drops of corn syrup, diluted with two to three parts of water, are added from time to time. The time in the reducer may be a minute or longer and should be controlled under the microscope. Further reduction will take place in the alcohol to which the tissue is taken prior to removal of collodion.

(b) *Foot*<sup>39</sup> also offers a modification of Cajal for sections on the slide:

(A) Fix for twenty-four hours in Carnoy's fluid (p. 466).

Imbed, section, mount on slides and deparaffinize.

(B) Refix for twelve hours in:

Pyridine.....	2 parts
Glycerine.....	1 part

(c) Wash.

(d) Silver in 10 per cent aqueous solution of silver nitrate for twelve hours on the incubator.

(E) Wash in two changes of distilled water.

(F) Reduce for twenty minutes in 100 c.c. of 5 per cent neutral formol containing 0.5 gm. of pyrogallol.

Wash in tap water and tone in 1:500 aqueous gold chloride for five minutes. Reduce the gold by immersion for five minutes in 100 c.c. of 1 per cent neutral formol to which are added 2 gm. of oxalic acid. Wash in tap water and fix for five minutes in 5 per cent aqueous sodium thiosulphate. Wash, dehydrate, clear and mount.

### c. Bielschowsky's Silver Methods.

The Bielschowsky procedure consists of: (A) fixation, usually in formol; (B) washing; (C) silvering; (D) washing; (E) treating with ammoniacal silver solution; (F) washing, and (G) reducing in formol. Variants in this procedure may be in the presence of a secondary fixation, the strength of the ammoniacal silver solution and the strength of the reducer. As a result of the reduction there follows a silver impregnation of the neurofibrils, axons and dendrites. Other structures, as fibers of connective tissue and neuroglia, may also be impregnated. Here as in all methods involving the reduction of gold and silver salts, the glassware must be physically and chemically clean, and the sections or blocks

<sup>39</sup> Foot, N. C. *Am. J. Path.*, 8: 769, 1932.



should be manipulated with glass rods, or with forceps having their points paraffin-coated.

The methods are applicable to thin pieces of tissue (not over 1 cm. thick) which have been recently fixed in formol, and also to similar pieces taken from brains or spinal cords which have been preserved for a long time, as long as ten years, in formol. They succeed both on human tissues from autopsy and on animal tissues. They are therefore of great value to the neuropathologist. The method is also valuable for motor and sensory nerve-endings, including those in the organs of special sense. The impregnation may be carried out either with frozen or paraffin sections, or with blocks of tissue which are later imbedded and sectioned in paraffin or collodion. The sections are always toned in gold chloride before being mounted.

*a. For Blocks of Tissue.* These methods permit serial sections.

(1) Without Secondary Fixation.

(A) Fix in 20 per cent neutral formol. Thin pieces cut from brains already fixed in formol may be used. Wash an hour or more.

(c) Silver in 2 per cent aqueous solution of silver nitrate in the dark, at room temperature for one to eight days according to the size of the blocks.

(D) Rinse in distilled water.

(E) Treat with ammoniacal silver solution for one and one-half to six hours with one change. This solution is made up as follows:

To 10 c.c. of 10 per cent aqueous solution of silver nitrate, 5 drops of 40 per cent sodium hydroxide are added. The precipitate thus formed is nearly all dissolved by the addition of concentrated ammonia drop by drop, the solution being well shaken after each drop is added. Distilled water is then added to bring the quantity up to 20 c.c. This solution must be made up just before using. Immediately after using the dishes should be thoroughly cleaned and kept for this mixture only. Ammoniacal silver when dry is highly explosive.

(F) Rinse in distilled water.

(G) Reduce in 20 per cent neutral formol twelve to twenty-four hours.

The blocks are then washed in distilled water, dehydrated in graded alcohols, quickly imbedded in paraffin, sectioned and deparaffinized.

From water the sections are toned for ten to thirty minutes in:

1 per cent aqueous gold chloride..... 2-5 drops

Distilled water ..... 10 c.c.

Glacial acetic acid..... 2-3 drops

Wash quickly in distilled water.

Fix in a 5 per cent solution of sodium thiosulphate for thirty to sixty seconds. Wash thoroughly in running water for several hours, dehydrate, clear in carbolyxol (1:10) and mount in balsam.

*Variant 1.* Agduhr gives a modification of this method which has been used successfully by many investigators.

- (A) Fixation in 50 per cent formol, five days or longer.
- (B) Wash thoroughly in distilled water.
- (C) Silver in a 3 per cent solution for six days.
- (D) Wash one hour in distilled water with many changes.
- (E) Treat for twenty hours with ammoniacal silver oxide made up as before in the following concentrations:

10 per cent silver nitrate.....	10 c.c.
25 per cent sodium hydroxide.....	20 drops
Distilled water .....	200 c.c.

Ammonia drop by drop until nearly all the precipitate disappears.

- (F) Wash for one hour in distilled water to which have been added 10 drops of acetic acid to each 100 c.c. Wash one hour in pure distilled water.
- (G) Reduce in 50 per cent formol, one to four days, renewed daily, and proceed as before.

## (2) With Secondary Fixation (Pyridine).

- (A) Fix for at least one week in 10 per cent formol.
- (B) Wash in running water four to six hours. Refix in pure pyridine three to four days. Wash in running water twelve to twenty-four hours, then in distilled water twelve to twenty-four hours.
- (C) Silver in 3 per cent solution, three to five days at 36°C.
- (D) Rinse in distilled water.
- (E) Treat for twenty-four hours with ammoniacal silver made up as in Method 1 in the following concentrations:

10 per cent silver nitrate.....	10 c.c.
40 per cent sodium hydroxide.....	5 drops

Ammonia drop by drop until precipitate disappears and two drops in excess. Distilled water up to 10 c.c.

- (F) Wash in many changes of distilled water fifteen minutes to two hours.
- (G) Reduce in 10 per cent neutral formol, ten to twelve hours, wash in distilled water and proceed as before.

## *b. For Sections.*

### (1) Frozen Sections.

- (A) Fix in 10-20 per cent neutral formol.
- (B) Wash an hour or more in running water. Prepare frozen sections as thin as possible compatible with handling, wash in distilled water (one to two hours) with several changes.
- (C) Silver in 2 to 3 per cent solution for twenty-four to forty-eight hours.
- (D) Rinse quickly in distilled water.
- (E) Treat for ten to twenty minutes, until the sections are deep brown, in ammoniacal silver solution made up as for pieces without secondary fixation: Further treatment is the same as before.

### (2) Deparaffinized Sections on the Slide. This offers the opportu-

nity of using more than one stain on the same block. Rogers<sup>40</sup> gives a number of variants of his own method which is a combination of Cajal and Bielschowsky techniques. The following variant gives consistent results with amphibian material.

(A) The blocks are fixed in Bouin's fluid or formol. The sections are refixed for twelve hours or more in ammoniated alcohol (95 per cent alcohol, 100 c.c. and ammonia 2 c.c.).

(B) Rinse in 80 per cent alcohol.

(C) Silver for twenty minutes or longer in the dark in 40 per cent aqueous solution of silver nitrate. Clothes and body should be protected from this solution.

(D) Rinse in distilled water, and cover sections with 20 per cent formol for two to five minutes.

(E) Drain off formol and cover slides with ammoniacal silver solution heated to 30° to 50°C., until sections turn a yellowish brown. The ammoniacal silver solution is made up by adding ammonia drop by drop to 4 c.c. of 20 per cent silver nitrate until the precipitate is formed and dissolved. Two drops of ammonia are added in excess and 4 c.c. of distilled water.

The sections are then toned for ten to fifteen minutes in:

Gold chloride .....	1 gm.
Distilled water .....	300 c.c.
Glacial acetic acid .....	60 drops

Wash in distilled water. Fix in 5 per cent sodium hyposulphite for five minutes. Wash in running water, clear and mount.

Rogers also says the sections may be reduced in 20 per cent formol before toning.

For nerve endings, he leaves the sections in the 40 per cent silver for three to five weeks, and carries out the reduction directly in 20 per cent formol, without the intermediate step of ammoniacal silver.

Another variation is to leave the sections for thirty minutes in 0.5 to 1 per cent aqueous solution of acetic acid before silvering for five to seven weeks.

Foot<sup>41</sup> uses the Rogers's procedure after Carnoy's fixation and finds it applicable for demonstrating terminal nerve endings and non-medullated fibers in the central nervous system. His formula for Carnoy's fluid is:

Absolute alcohol .....	6 parts
Chloroform .....	3 parts
Glacial acetic acid, added just before using .....	1 part

<sup>40</sup> Rogers, W. M. *Anat. Rec.*, 49: 81, 1931.

<sup>41</sup> Foot, N. C. *Am. J. Path.*, 8: 769, 1932.

3. **Internal Reticular Apparatus of Golgi (Golgi Net).** The material should be fresh, not more than two hours after death. Fetal and young mammalian tissues are preferable. The original Golgi method has been largely replaced by the following and other modifications. In successful preparations the Golgi net shows as a black network on a bright yellow background.

*Cajal's* uranium-formol method, for Golgi net.

1. Fix pieces 2 to 2½ mm. thick for eight to twenty-four hours in the following:

Uranium nitrate .....	1 to 2 gm.
Neutral formol, full strength .....	15 c.c.
Distilled water .....	85 c.c.

The duration of fixation should vary with the material; often in young animals a few days old it is necessary to leave for twenty-four to thirty-six hours.

2. Rinse in distilled water less than a minute.  
3. Transfer to 1½ per cent silver nitrate (if the pieces are very small, 0.75 per cent) for thirty-six to forty-eight hours.

4. Rinse for some seconds in distilled water.

5. Reduce eight to twelve hours, in:

Hydroquinone .....	1 to 2 gm.
Neutral formol, full strength .....	15 c.c.
Distilled water .....	85 c.c.
Anhydrous sulphite of sodium .....	0.15 gm.

6. Wash briefly in running water. Dehydrate in graded alcohols, leaving the pieces not more than one to two hours in the 90 per cent and absolute alcohols. Imbed in collodion or paraffin.

*Da Fano's* modification is to use cobalt nitrate instead of uranium nitrate as the fixing agent, making it as follows:

Cobalt nitrate .....	1 gm.
Neutral formol .....	15 c.c.
Distilled water .....	100 c.c.

The best coloration of the Golgi apparatus is obtained in the cat, dog and rabbit, in animals fifteen to twenty days old. By augmenting the quantity of the formol (up to 20 per cent) in the fixing fluid, as proposed by Penfield,<sup>42</sup> or by excluding the sodium sulphite in the reducer, splendid coloration has also been obtained with adult tissues. The zone of useful reaction is always small (0.2 to 0.5 mm.), for it is not possible to use pieces thicker than 3 mm. The sulphite is not a necessity, but it appears that a little alkalinity favors the coloration of the Golgi net. One may counterstain with hematoxylin or a basic aniline dye.

<sup>42</sup> Penfield, W. G. *Anat. Record*, 22: 57, 1921.

When the coloration is intense but somewhat granular one may try adding to the fixative ethyl or methyl alcohol, thus:

Uranium nitrate.....	1 gm.
Alcohol, ethyl or methyl.....	30 c.c.
Neutral formol.....	15 to 20 c.c.
Distilled water.....	80 c.c.

With this formula successful preparations show the Golgi net dark gray on a bright yellow background.

The Kopsch method and others described under Cytological methods are equally useful here.

4. **Mitochondria.** The methods used with the other tissues are applicable also to the nervous system (p. 265). The Champy-Kull method gives good results with mammalian nervous tissues (p. 269).

5. **Glycogen.** The glycogen in the nerve-cells may be fixed by alcohol at any concentration from 67 per cent to absolute, but it is soluble in alcohol less than 67 per cent. Plenty of alcohol should be used, about 50 times the bulk of the tissue. Material may be decalcified, after fixation in alcohol, with 3 per cent nitric acid in 67 per cent alcohol. Another fixing fluid is picric-alcohol, made of 500 c.c. of 67 per cent alcohol with 1 gm. of picric acid. Leave in this fluid for twelve to twenty-four hours, in 67 per cent alcohol twelve to twenty-four hours, and in 82 per cent alcohol for a day or more. Imbed in paraffin or in combined paraffin and collodion, and cut sections at  $10\mu$  to  $15\mu$ .

Stain with iodine. A useful formula is:

95 per cent alcohol.....	150 c.c.
Distilled water.....	150 c.c.
Iodine, crystals.....	1.5 gm.
Potassium iodide.....	3 gm.
Sodium chloride.....	1.5 gm.

For staining spread the paraffin sections with the staining fluid instead of water, and leave for two to three minutes. If the paraffin is not melted off the sections may be restained at any time. Such preparations are useful only for low-power observation. They may be mounted in vaseline as follows: Stain without removing the paraffin, then dry for half an hour on top of the oven or in the air. When thoroughly dry, dissolve off the paraffin in xylol and cover with yellow vaseline. It is best to seal around the margin of the cover glass with shellac. The stain lasts two to ten years. The sections may also be mounted with heated balsam, without covers. This is better for study with the highest powers of the microscope, but the stain does not last as long as with the vaseline (Gage).<sup>43</sup>

<sup>43</sup> Gage, S. H. *J. Comp. Neurol.*, 27: 451, 1917.

### 6. Trophospongium of Holmgren.

Several methods may be used. Ross,<sup>44</sup> studying nerve-cells of the abdominal ganglia of the crayfish (*Cambarus*) used Bensley's acetic-osmic-bichromate acid fuchsin method, or fixed in Zenker's fluid and stained with Mallory's connective tissue stain.

Penfield,<sup>45</sup> to show that the trophospongium and the internal reticular apparatus of Golgi were separate structures, stained the former with iron hematoxylin.

**7. Centrosomes.** For the demonstration of centrosomes in the nervous system, several methods are used. Flemming's fixing solution or Allen's chromic-urea modification of Bouin's fluid followed by Heidenhain's iron hematoxylin is especially good.

Hatai<sup>46</sup> demonstrated their presence in certain nerve-cells of the albino rat. In addition to other methods, he devised one of his own which gave him good results:

Mercuric chloride, saturated solution in formol.....	30 c.c.
Glacial acetic acid.....	50 c.c.
Physiological salt solution.....	15 c.c.

After six to twelve hours' fixation, the thin pieces were washed in running water for four to five hours, transferred to 30 per cent alcohol, and then by graded alcohols were dehydrated and imbedded in paraffin. For staining he used toluidine blue or thionin in saturated aqueous solution; cleared in xylol, mounted in balsam. By this method centrosomes were shown in the large pyramidal cells of the cerebral cortex, Purkinje cells and spinal ganglion cells of the adult albino rat, and also in the ventral horn cells of the spinal cord and in the cells of the nucleus dentatus of the newborn rat.

Rio-Hortega<sup>47</sup> has successfully applied to the study of centrosomes the neuroglia stain with tannin-ammoniacal silver, which he terms his "first variant of the method of Achúcarro" (p. 509).

**8. Pigment.** The endogenous pigment in nerve-cells is usually of a bright golden color, in the form of granules, generally massed in one region of the cell-body, and is readily recognized by its natural coloration.

## VIII. Normal Myelin Sheath

**1. Weigert's Myelin-sheath Methods.** These methods are used principally on formol fixed tissues of the central nervous system to dem-

<sup>44</sup> Ross, L. S. *J. Comp. Neurol.*, 25: 523, 1915.

<sup>45</sup> Penfield, W. G. *Brain*, 43: 290, 1921.

<sup>46</sup> Hatai, S. *J. Comp. Neurol.*, 11: 25, 1901.

<sup>47</sup> Rio-Hortega, P. *Trab. Lab. Investig. Biolog. Univ. de Madrid*, 14: 117, 1916.

onstrate the fiber-tracts and to show the arrangement of the gray and white matter. Proper fixation and mordanting are necessary for the success of the staining. The nervous tissue, either in the form of the entire brain or cord or small portions of them, or of short pieces of peripheral nerves, is fixed in 10 per cent formol. The duration of fixation for small pieces is a week or more; for the entire human brain one to two months or longer. If the tissues are to be set aside for further preparation at a later time it is best to keep them in neutral 10 per cent formol.

The following modification has been found useful on both human and animal tissues. This requires the employment of four special solutions: the primary mordant (A), the secondary mordant (B), the hematoxylin stain (C) and the differentiating fluid (D) made respectively as follows:

A. Primary mordant (Weigert's rapid mordant)

Potassium dichromate .....	5 gm.
Fluorochrome ( $\text{CrF}_3 \cdot 4\text{H}_2\text{O}$ ) .....	2 gm.
Distilled water (boiling) .....	100 c.c.

B. Secondary mordant (copper mordant, Weigert's neuroglia mordant)

Copper acetate .....	5 gm.
Acetic acid, 30 per cent or 36 per cent .....	5 c.c.
Fluorochrome .....	2.5 gm.
Distilled water .....	100 c.c.

In making this solution, boil the fluorochrome and water in a covered vessel, turn off the gas, add the acetic acid, then the copper acetate, stir briskly until the latter is dissolved and allow to cool. The solution remains clear.

C. Hematoxylin stain

10 per cent solution of hematoxylin in abs. alc. (stock sol.) ..	10 c.c.
Lithium carbonate (saturated aqueous sol.) .....	1 c.c.
Water .....	90 c.c.

It is very important that the stock solution of hematoxylin should be well ripened (two months or more, six months preferably), but the staining fluid should be made up just before using.

D. Differentiating fluid

Potassium ferricyanide .....	2.5 gm.
Sodium biborate (borax) .....	2.0 gm.
Distilled water .....	100.0 c.c.

After fixation the blocks of tissue are washed in running tap water overnight, before being placed in the primary mordant. Here they remain one week or longer, depending upon their size. After washing they are passed into the secondary mordant for one to several days. The blocks are then washed in water, dehydrated in graded alcohols and imbedded in collodion. Sections are usually cut at  $25\mu$  to  $50\mu$ . They are then placed in the staining solution overnight (twenty to twenty-four hours) where

they become a dense blue-black color. After staining the sections are washed thoroughly in several changes of tap water, and left in the water overnight. They are then placed in the differentiating fluid and the decolorizing process watched. The color gradually leaves the gray matter, but is retained in the myelin sheaths of the white matter, so that the distinction between the gray and the white matter becomes more and more distinct. The time for this process is usually between twenty minutes and an hour. The differentiation is stopped by transferring the sections to water.

After differentiation the sections are to be thoroughly washed in tap water (several changes and leave overnight), dehydrated in alcohols, cleared in carbol-xylol followed by xylol, and mounted in balsam.

*Variant 1.* Paula Meyer<sup>48</sup> in Edinger's laboratory used potassium bichromate without fluorochrome as a primary mordant and cut sections before the secondary mordant. Sections were stained overnight or twenty-four hours, in Weigert's iron hematoxylin, prepared as follows:

Distilled water .....	90 c.c.
10 per cent solution of hematoxylin in abs. alc. (ripened) ..	10 c.c.
Solution of ferric chloride, U.S.P. ....	4 c.c.

After differentiating the sections were placed in lithium carbonate, changed repeatedly for twenty-four hours. The myelin sheaths are stained black, whereas with the lithium hematoxylin they are stained blue.

*Variant 11.* For brains of albino rats Craigie<sup>49</sup> found more satisfactory results with an earlier form of the method. Fixation was in Müller's fluid, which was renewed two or three times during the first week, and then left unchanged in the dark for two months or more. The tissue was then placed in distilled water for several hours before dehydrating and imbedding in paraffin. The sections were affixed to the slide by the water-albumen method, and when dry were passed through xylol and absolute alcohol, and then coated with a layer of very thin collodion (0.5 per cent). This film hardens quickly in the air. The slides were then passed down through graded alcohols to water. Mordanting was effected by placing the slides upside down (supported on small pieces of glass) in a half-saturated solution of copper acetate, at above 35°C., overnight. After rinsing in distilled water they were stained for four to six hours, in lithium-hematoxylin, the slides being horizontal with the sections underneath again. After washing they were differentiated with the borax-ferricyanide solution. They were then washed in running water for twenty-four hours, dehydrated, cleared and mounted in Canada balsam.

<sup>48</sup> Meyer, P. *Neurol. Centralb.*, No. 7, 1909.

<sup>49</sup> Craigie, E. H. *The Finer Anatomy of the Central Nervous System of the Albino Rat*. Toronto, 1925.



*Variant III. Pal-Weigert Method, Modified.* The following method is one used at the Netherlands Central Institute for Brain Research, Amsterdam, by Dr. Ariens Kappers, and is applicable both to human and comparative material. With the Pal variation the differentiation between the myelinated fibers and the surrounding tissues may be carried to a greater degree than in the original methods of Weigert. For some purposes this is desirable, but for others it may not be an advantage. In the original Weigert the cell-bodies are brownish in color and in a general survey of a region, this adds to the completeness of the picture.

Fixation is in 10 per cent formol, one week or longer. This is followed by immersion in 3 per cent potassium dichromate solution (primary mordant) for one to four weeks, according to the size of the blocks. The tissues are then washed well in running water, dehydrated in graded alcohols, imbedded in collodion and cut at  $25\mu$  to  $75\mu$ . If the sections are being kept serially they should be converted into films at this stage on large slips. The individual sections or films are then placed for one to two hours in 100 c.c. of 0.5 per cent chromic acid to which has been added 1 c.c. of 1 per cent solution of osmic acid (secondary mordant). Rinse sections in water briefly, and stain twelve to twenty-four hours in the following hematoxylin stain (Kultschitsky's hematoxylin) freshly made up:

Ripened 10 per cent solution of hematoxylin in abs. alc...	10 c.c.
Acetic acid, glacial .....	1 c.c.
Distilled water .....	100 c.c.

This solution may be used either cold or warm. If kept in an incubator at not over  $37^{\circ}\text{C}$ . the color becomes very intense, but the drawback is that the sections tend to become brittle.

Wash in water until the color ceases to come out (not more than an hour is necessary), then begin the differentiating process. Immerse in 0.5 per cent potassium permanganate in water for thirty seconds, rinse in water, and pass into the following solution (Lustgarten's):

1 per cent potassium sulphite, in distilled water .....	100 c.c.
1 per cent oxalic acid, in distilled water .....	100 c.c.

Mix the two solutions just prior to use. The differentiation must be watched closely, for it may proceed very rapidly, usually requiring not more than five minutes. The sections may be taken out before the differentiation is complete, and after washing briefly in water, place them again in the permanganate solution for thirty seconds, rinse and then place in the Lustgarten solution as before. By this process the background of gray matter is bleached out while the white matter stands out in strong contrast with its dark blue color. It is important not to leave too long in the permanganate solution, for the staining depends on the presence of potassium dichromate, and if this is removed it cannot be replaced, even by a renewed treatment with potassium dichromate. For this reason it is

better to keep the sections in the differentiating fluids for a short time only, and to repeat the processes even several times, in order to control the reactions.

The sections, after being rinsed in tap water, are then transferred to distilled water, to which has been added a little lithium carbonate (water 500 c.c.; sat. aq. lith. carb., 1 c.c.) where they remain for twenty minutes; or they may be left overnight. Dehydrate in graded alcohols, clear in carbol-xylol followed by xylol and mount in balsam.

*Variant iv.* Smith and Quigley<sup>50</sup> have developed a rapid method for staining myelin sheaths in sections. It is applicable to frozen, paraffin or collodion sections of normal or pathological material. The myelin sheaths are stained a deep blue and the background is either colorless or slightly tinted, depending upon the degree of differentiation. The structure of the cell nuclei is clearly shown.

(1) Mordant sections for fifteen minutes in 4 per cent aq. sol. of ferric ammonium sulphate ( $\text{FeNH}_4(\text{SO}_4)_2$ ).

(2) Rinse briefly in 70 per cent alcohol.

(3) Place sections in the following fluid, which first stains then differentiates:

10 per cent hematoxylin in absolute alcohol ..... 10 c.c.

Distilled water ..... 88 c.c.

Glacial acetic acid ..... 2 c.c.

The 10 per cent hematoxylin solution may be either freshly made or older, but preferably not over three months old.

Keep around 55°C. until the proper degree of differentiation occurs; usually between thirty and sixty minutes are required, depending upon the thickness of sections. A convenient method of warming the staining-differentiating fluid is to place it in a Petri dish on a slide warming table.

(4) Place in a saturated aqueous solution of lithium carbonate for three minutes.

(5) Rinse briefly in tap water. Counterstain if desired, dehydrate, clear and mount.

**2. Iron Hematoxylin Myelin Stain.** This method of staining myelin is especially useful on old formol-fixed material, which may be refractory to other methods, but is applicable also to fresh material following nearly any fixation. It is also useful when more than one stain is desired from the same block. The Morgan<sup>51</sup> procedure is as follows:

(1) Fix parts of central nervous system in formol, alcohol-formol, alcohol, Bouin's, Carnoy's, Flemming's, etc. Dehydrate, imbed in either paraffin or collodion, and section.

(2) Mordant sections two hours or less in 4 per cent aqueous solution of iron alumferric ammonium sulphate [ $\text{Fe NH}_4(\text{SO}_4)_2$ ].

<sup>50</sup> Smith, W. K., and Quigley, B. Pers. comm.

<sup>51</sup> Morgan, L. O. *Anat. Record*, 32: 283, 1926.

(3) Stain three to eight hours in: ripened 10 per cent solution of hematoxylin in absolute alcohol, 5 c.c., distilled water, 95 c.c.

(4) Destain in 2 per cent iron alum for a few minutes until the larger fiber tracts become well outlined.

(5) Rinse in tap water.

(6) Complete the differentiation in 0.5 per cent hydrochloric acid; in a few seconds the background becomes colorless and the fibers well differentiated.

(7) Wash the sections in running water, for at least an hour, to remove the acid and alum.

(8) (Counterstain with well-ripened neutral red, if desired.)

(9) Dehydrate, clear in xylol and mount in balsam.

In successful preparations the myelinated fibers are stained a dark blue, while the background is clear and white. In destaining with the 2 per cent alum, the process should be stopped by placing the sections in tap water before all the blue of the hematoxylin has been extracted from the background; and the treatment with the hydrochloric acid should be very brief and should be carefully watched. When the proper stage has been reached the decolorizing is stopped by transferring the sections to tap water.

The Weil<sup>52</sup> procedure may be used for sections imbedded either in paraffin or collodion, but the best results are obtained with loose collodion sections, cut at 25 or 30 microns. If sections are not to be stained immediately after cutting, they should be stored in 50 per cent alcohol.

(1) Wash in distilled water.

(2) Mordant in a 5 per cent aqueous solution of potassium dichromate from five to ten minutes. Freshly imbedded material does not require mordanting. Old formol-fixed material or sections which have been in alcohol for a long time must be mordanted.

(3) Rinse in tap water, two changes.

(4) Stain for fifteen to thirty minutes according to thickness of sections in the following solution in the oven at 50°C.:

4 per cent aqueous solution of iron alum .....	50 c.c.
1 per cent solution of hematoxylin .....	50 c.c.

The hematoxylin is made up by adding 45 c.c. of distilled water to 5 c.c. of 10 per cent solution of hematoxylin dissolved in absolute alcohol (we use freshly dissolved hematoxylin). Do not filter this stain.

(5) Wash twice in tap water.

(6) Differentiate in a 4 per cent aqueous solution of iron alum until the gray matter and degenerated areas can be just distinguished.

(7) Wash three times in tap water.

<sup>52</sup> Weil, A. 1933. Pers. comm.

(8) Complete the differentiation in one-half strength Weigert's differentiating fluid (p. 470).

(9) Wash twice in tap water.

(10) Place sections in ammoniated tap water for thirty seconds: water 100 c.c., concentrated ammonia 6 drops.

(11) Wash twice in tap water, dehydrate in 95 per cent alcohol and absolute alcohol, clear in xylol and mount.

This method has been used successfully on serial collodion sections affixed to the slide. The nuclei may stain, but they do not interfere with the picture.

3. The osmic acid methods described under isolation of the nerve fibers may also be used for the study of the myelin sheath.

### IX. Degenerating Myelin Sheath

1. **Marchi Method.** When a nerve-fiber is cut across or severely injured, the degeneration of the distal portion of the axon is accompanied by changes in chemical constitution and structural form of the myelin. The myelin loses its uniform distribution and becomes aggregated into little globules of varying sizes, along the neurokeratin framework. At the same time appears an acid, oleic acid, which stains readily with osmic acid. By using a solution containing potassium dichromate before, or at the same time as, the osmic acid a differential staining reaction on normal and degenerating myelin is obtained. The normal myelin is quickly oxidized by the potassium dichromate, and thus cannot be oxidized by the osmic acid. The normal myelin therefore remains unstained, while the fatty acid of the degenerating myelin is blackened.

#### Summary of Marchi Method.

(1) Fix blocks of tissue in 3 per cent potassium dichromate for three weeks, changing several times. The time required may vary from one to five weeks.

(2) Cut out the parts desired, 2 to 3 mm. thick, and place directly in the Marchi fluid for one to three weeks, renewing the solution weekly.

3 per cent potassium dichromate ..... 2 parts

1 per cent osmic acid ..... 1 part

Keep this solution tightly covered.

(3) Wash in running water twenty-four to forty-eight hours, dehydrate and imbed in collodion. Cut sections 20 to 50 $\mu$ , according to purpose, and mount in balsam with or without a coverglass. Carbol-fuchsin, safranin or van Gieson's stain may be used as a counterstain.

The degenerating myelin shows as black dots and flakes on a yellowish or brownish background of normal fibers, neuroglia and nerve cell

bodies. Besides the black particles of the degenerating myelin, an irregular precipitation may also occur, and it is necessary to be on one's guard in interpreting the appearances. Special care must be taken in handling and removing the fresh tissues, lest the degenerating myelin be displaced.

As the process of degeneration is a progressive one, ending in total degeneration or regeneration, there is a limited time during which the myelin droplets are most numerous. Usually the material is taken between the tenth and the twenty-fifth day in mammals, and between thirty and forty days in cold-blooded animals.

Mettler<sup>53</sup> uses the following procedure on the brains of rabbits, cats and dogs. The animal is killed by illuminating gas and bled from the heart. The brain is exposed and immersed in situ in 50 per cent neutral formol, twenty-four hours, after which time pieces 4 mm. are cut out and placed in an abundance of 3 per cent potassium dichromate for two weeks in the dark. The potassium dichromate should be aged for three months, but should not be a green color. The solution should be changed when it becomes discolored. All further steps until the tissue is imbedded are carried out in the dark.

The tissue is rinsed and placed in ample Marchi fluid made up of four parts of 3 per cent potassium dichromate and one part of 1 per cent osmic acid. More osmic acid should be added if the odor disappears and the solution should be changed if it turns black. Leave here for two to three weeks. The tissues are then washed one to two days, depending on their size, dehydrated and imbedded.

Swank and Davenport<sup>54</sup> recommend for the spinal cord and medulla of cat and rabbit, fixation in 100 c.c. of 10 per cent neutral formol to which has been added 1 gm. of potassium chlorate, for forty-eight hours. Without washing, the tissues are placed directly in the Marchi fluid, with or without the addition of 2 per cent acetic acid for one week.

Papez and Freeman<sup>55</sup> use the simple procedure of fixing in formol overnight, cutting out pieces and refixing them in 3 per cent potassium dichromate for ten days, after which time an equal amount of 1 per cent osmic acid is added to the solution and the tissue left there for ten days.

## X. Methods for Nerve Endings

Nearly all the silver methods described under the methods for demonstrating neurofibrils (Cajal, Bielschowsky) impregnate the peripheral

<sup>53</sup> Mettler, F. A. *Stain Technology*, 7: 95, 1932.

<sup>54</sup> Swank, R. L., and Davenport, H. A. *Stain Technology*, 9: 129, 1934.

<sup>55</sup> Papez, J. N., and Freeman, G. L. *J. Comp. Neurol.*, 51: 409, 1930.

nerve endings. For the best results material should be fresh and preferably from young animals.

*Gold Chloride Method.* For many years gold chloride has been used to demonstrate the motor nerve endings in striated muscle, the myenteric plexus of the intestine, the nerves of the cornea, etc.

Garven<sup>56</sup> employed the modification of Ranvier's gold chloride method as used in Golgi's laboratory. It is successful with motor nerve endings in striated muscle. It is advisable to use short muscles, as the intercostals of small animals.

(1) Immerse small pieces in 25 per cent aqueous solution of pure formic acid, for ten to fifteen minutes. Use only enough solution for the complete immersion of the pieces. The pieces should be teased a little in the solution.

(2) Take pieces from the acid solution and place on a clean folded towel or duster, cover the pieces with another fold of the towel, and press gently to absorb as much acid as possible.

(3) Transfer to 1 per cent solution of gold chloride, just sufficient to cover the pieces, for twenty minutes at most, shaking the pieces around in the dish several times. Keep dish covered with blue or yellow glass. No iron instruments must be allowed to come in contact with this or the subsequent baths; use either bone-pointed forceps or paraffin-coated points of metal ones.

(4) Mop as in (2).

(5) Transfer to 25 per cent formic acid, using just enough to cover the pieces, and keep in absolute darkness for twenty-four hours.

(6) Mop as in (2).

(7) Preserve in pure glycerin.

Nerve fibers are intensely purple or black. Good preparations keep five years or more, becoming clearer with time. Cleanliness as to dishes and instruments is important.

*Intra-Vitam Methylene Blue Method.* This was introduced by Ehrlich and has been used by many investigators. The composition of the dye is very important in attaining good results. It is believed that the purer the methylene blue, the more successfully it can be used for vital staining. The U.S.P. zinc-free methylene blue is always recommended,<sup>57</sup> and it is best to specify dye certified by the Commission on Standardization of Biological Stains. The method is much more applicable to the staining of the peripheral ganglia (spinal, cerebral, sympathetic, parasympathetic), peripheral nerves and nerve endings than to the staining of the elements of the central nervous system, although the latter may also be stained by this method.

The diluted stain is applied directly to the fresh tissues. This may be

<sup>56</sup> Garven, H. S. D. *Brain*, 48: 380, 1925.

<sup>57</sup> Biological Stains. Geneva, N. Y., 65, 1929.

done in several ways: (1) by moistening thin pieces of the excised tissue with the fluid, (2) by perfusing the blood vessels of the whole animal or of the region desired, (3) by injecting into the natural cavities of the body, as into the peritoneal cavity, into the pleural cavity or into the lungs through the trachea, (4) by injecting into the loose connective tissues of the part to be examined.

The dilution of the staining fluid varies with the material, the method of applying the stain and the purposes of the study. The time required for the staining is usually between fifteen and thirty minutes but may be as long as three or four hours, and has to be determined by trial.

After the nerves are stained blue the tissues may be studied at once, but as the stain is transitory it is preferable to fix it so that the tissue may be imbedded and sectioned. Ammonium molybdate is used for the fixation of the stain in the tissue, using an 8 per cent solution in physiological salt solution or Ringer's solution, for one-half hour to overnight. The tissue is then washed in running water for an hour and dehydrated rapidly, in order to avoid losing the color. From the water the tissue is passed into 95 per cent alcohol for ten to sixty minutes, and then into absolute alcohol for one to two hours.

In successful preparations the ganglion cells with their processes, myelinated and unmyelinated, and their end-arborizations are colored blue. Other tissues should be nearly colorless unless injured by pressing or cutting, in which condition they become deeply colored. Elastic fibers and connective tissue cells may assume a blue color, and may be then mistaken for nerve elements if the latter are not in evidence.

The peculiarity of the reaction of the dye in this method is that the maximum staining lasts only a short time in the fresh tissue. This is explained by the fact that the blue compound becomes colorless when the oxygen content of the tissue becomes lessened. For this reason, when in a fresh preparation the color is seen to be fading, the color may sometimes be restored by removing the coverglass and allowing the oxygen of the air to have access to the tissue.

#### (1) Method by Local Application.

(1) Place thin pieces of fresh tissue on a slip or in a shallow dish (the bottom of the latter may be covered with a thin layer of glass-wool on which the tissues rest) moistened with the staining solution, e.g., 1 part of methylene blue to 1000 to 2000 parts of physiological salt solution.

(2) Drop on enough staining fluid from time to time to keep the tissues moist, with a film of stain over them.

(3) After fifteen minutes the tissues are examined with low magnification and at short intervals thereafter until the nerves are colored blue.

(4) Fix the stain by placing the tissues in cold 8 per cent ammonium molybdate solution made up with physiological salt solution or Ringer's fluid.

(5) After washing in cold water and dehydration the tissues may be either cleared in xylol and mounted in balsam or imbedded in paraffin and sectioned. The alcohols also should be cold and kept in the refrigerator, at several degrees above 32°C., during the dehydration of the tissues. The sections may be lightly counterstained.

A variation of this was used by Cole,<sup>58</sup> who immersed the entire digestive tube of the frog in a 1:1000 solution for one hour. Small segments were then cut out, flattened between two slips and examined under low power. If the stain was not satisfactory the tissues were replaced in the stain; if satisfactory, they were fixed in 8 per cent molybdate in Ringer's, for a half hour. The tissues were placed in a muslin bag and washed for an hour. The excess water was squeezed out on a towel and the tissues placed in ice-cold 95 per cent alcohol for ten minutes, then in ice-cold absolute alcohol for ten minutes. They were cleared in xylol, if thin, or in cedar oil if thick, and mounted in balsam.

Large pieces as of skin or thin membranes may be stretched and pinned to a flat piece of cork which is then floated inverted on the staining fluid. If the cork has a large opening made in it, the tissue may be kept on the cork while the differentiation is observed.

(2) Method by Perfusion and Injection.

(1) Insert a cannula into the heart or aorta, or into the main artery of the part to be examined. Inject the filtered dilute solution of the dye (for mammalian tissues 1 to 10,000 or even more dilute) until the part has a distinctly light blue color.

(2) Leave undisturbed for a quarter of an hour, after which time thin pieces or slices of the tissue are removed and placed on a slip or in a dish moistened with the dye.

(3) These are examined under a low power of the microscope, without cover glass every two or three minutes until the nerve cells, nerve fibers or nerve endings seem satisfactorily stained.

(4) Fix the stain in 8 per cent ammonium molybdate, one-half hour to overnight, and continue as in the preceding summary.

In the case of large fetuses, as of pig, the injection may be made through one of the umbilical arteries. Langworthy<sup>59</sup> used this method, after first washing out the blood with physiological salt solution. For the dye he used 1:2000 methylene blue, rendered slightly alkaline with ammonium hydroxide. The tongue was removed and thin slices cut with a sharp knife. The sections were spread out on slips and placed in an

<sup>58</sup> Cole, E. C. *J. Comp. Neurol.*, 38: 375, 1925.

<sup>59</sup> Langworthy, O. R. *J. Comp. Neurol.*, 36: 273, 1924.



observation warm-box (as used for examining tissue cultures) until the nerve endings appeared. The musculature of the tongue had to be separated in order to admit of free oxidation, and even then there were always large areas where the stain was not oxidized.

For the study of the nerve endings in the lungs of rabbits, Larsell<sup>60</sup> used warm 1:2000 methylene blue in Locke's solution, or in 0.9 per cent NaCl solution, and filled the lungs through the trachea, or injected the fluid through the pulmonary vessels. The lungs were allowed to remain undisturbed in the thorax for ten minutes. The excess of fluid was allowed to run out of the lungs in the cases in which they had been filled. Then, after both methods of application of the stain, the lungs were alternately inflated and deflated with air through the trachea, at the rate of 12 to 15 times per minute. It was found that twenty to twenty-five minutes was the optimum time for continuing the oxidation of the stain in the lung tissue in this manner.

The lungs were extirpated and filled with 8 per cent cold ammonium molybdate to which had been added 2 to 5 drops of 1 per cent osmic acid per 100 c.c. of ammonium molybdate. They were immersed in a solution of the same composition and left in it overnight in the cold. After being washed in running tap water for one hour the lungs were filled with 95 per cent alcohol and immersed in 95 per cent alcohol for an hour, the alcohol within the lungs being changed several times during the hour. They were next placed in absolute alcohol for several hours to overnight, to complete the dehydration. Suitable pieces were cut out, 2 to 4 mm. thick, cleared in xylol, imbedded in paraffin, and sectioned at 25 $\mu$  to 100 $\mu$  thick. He found that the lungs injected through the pulmonary vessels showed the bronchial endings better, and that those injected through the trachea showed the endings in the walls of the pulmonary vessels more clearly.

<sup>60</sup> Larsell, O. J. *Comp. Neurol.*, 33: 105, 1921.

# SILVER METHODS FOR BOUTONS TERMINAUX AND NEUROFIBRILS

WILLIAM C. GIBSON

Introduction 481. Gelatin embedding, for silver stains 482. Rio-Hortega's double impregnation for frozen sections 483. Cajal's silver nitrate method for frozen sections 484. Block silver methods 485. Solutions 486.

## I. Introduction

The microscopic anatomy of the synapses of the central and peripheral nervous systems has reappeared as a subject worthy of fresh study. Once the keynote of the controversy over the discontinuity of the nervous system, the terminations of axones—*boutons terminaux*—have today become important for the help which they afford in the tracing of fiber tracts. Experimental interruption of a pathway in the nervous system causes marked degenerative changes in the boutons concerned, within a short time.

While it has been known since the beginning of the century that the axones of nerve cells end in minute ring-like structures upon the surface of other cells or their dendrites, it has been difficult to find histological methods for staining the boutons with clearness and reliability. The interneuronal connections of the spinal cord can be stained with facility and were very accurately demonstrated by Cajal<sup>1</sup> in 1903. Hoff (1932)<sup>2</sup> has described the changes which these synaptic structures undergo after dorsal root or pyramidal tract section.

The *methods of Cajal and Rio-Hortega* described in the following pages are applicable to the cerebrum and cerebellum, tissues in which a high myelin content has made the staining of neurofibrils with silver a difficult proposition. It is indeed fortunate for the experimental worker using rather capricious and empirical silver methods, that degenerating neurofibrils, and therefore boutons, have an increased affinity for silver salts.

The chief precaution in the use of the stains to be described is that distilled water and chemically clean glass must be used, and at all times metal contacts must be avoided. Frozen sections are passed through small

<sup>1</sup> Cajal, S. Ramón y: Un Sencillo método de coloración selectiva del retículo protoplásmico. *Trab. Lab. de Invest. Biol. Univ. Madrid.* 2: 129-221, 1903.

<sup>2</sup> Hoff, E. C. Central nerve terminals in mammalian spinal cord and their examination by experimental degeneration. *Proc. Roy. Soc. London.* B, 111: 175-188.

12 c.c. pyrex cups by means of a thin glass "hockey-stick." The formol, pyridine, and pyrogalllic acid used should be chemically pure, and not of the "commercial" quality. The ammonia is the concentrated solution, specific gravity 0.88. The manipulation of small sections in the staining cups is immeasurably easier if a table-top of black and white checker-board linoleum is available. A very small Bunsen burner is used to heat the solutions until hot to the touch. The microscope should be close at hand for rapid examination of wet preparations.

To fix *nervous tissues* wash through the vessels concerned, with Ringer's solution, followed immediately by a dilute solution of the fixative. Specimens should not be fixed in the same jar with other types of tissue. In the frozen section techniques which follow, it may be advantageous to freeze in situ, with liquid carbon dioxide, small objects such as *sympathetic ganglia*. These should be removed while solid, placed on the freezing microtome, and the sections cut directly into 10 per cent formol. If it is necessary to demonstrate oligodendroglia, astrocytes or microglia, as well as neurofibrils and boutons, in a specimen of nervous tissue, fix first in formol-ammonium-bromide solution (p. 505). The block to be examined with the neurofibrillar techniques should then be placed in formol.

It need scarcely be pointed out that in the block methods the worker risks everything, and has little control over the impregnation. "Patchy" staining and "burning" can be reduced to a minimum by wrapping the blocks of tissue in filter paper, by shielding the bottles of silver nitrate from the light, and by renewing the solution after the third day of incubation. The frozen methods offer a greater degree of control during the staining, and shorten the time of impregnation from several days to a few minutes.

## II. Gelatin Embedding

The following method is useful when very small objects such as *ganglia* are to be cut on the freezing microtome and carried through several silver solutions with a glass rod.

- (1) Fix tissues in 10-20 per cent formol. (For neuroglia, F.A.B.)
- (2) Wash in water for twenty-four hours, to remove all formol.
- (3) Place in "thin gelatin" for twenty-four hours at 37°C.
- (4) Place in "thick gelatin" for three to four hours only, at 37°C.
- (5) Refrigerate until solid. Block out with a knife.
- (6) Place in 10 per cent formol for twenty-four to forty-eight hours, to clear.
- (7) Cut on a freezing microtome and proceed with the techniques for frozen sections.

(8) In mounting these gelatin sections add 2 drops of ammonia and 5 drops of creosote to the alcohol used for dehydration. Creosote, blot dry, balsam, etc.

*Thin gelatin:*

- 0.5 gm. phenol crystals.
- 5 gm. white gelatin squares.
- 100 c.c. water.

*Thick gelatin:*

- Prepared as above, but with the gelatin increased to 10 gm.  
Squares of chemically pure white gelatin are required.

### III. Double Impregnation Method for Neurofibrils

This technique has been developed from a method first published by *Rio-Hortega*<sup>3</sup> in 1921. It consists essentially of a preliminary impregnation with a solution of silver nitrate, followed by treatment with silver carbonate.

*Fixation:* Tissues are best cut into small blocks or thin slices before fixation in a solution of 10 per cent formol in distilled water. To this may be added one drop of pyridine or one drop of ammonia, per cubic centimeter of formol. Both variants have proved useful in staining boutons in the superior cervical *sympathetic ganglion*. The optimum fixation period is ten days at 37°C. or one month in the cold. For use in cases where the tissue has been fixed in formol-ammonium-bromide solution (for neuroglia) a modification of the double impregnation technique for neurofibrils is appended below:

(1) Cut fixed material on the freezing microtome at 12-15 $\mu$ . Wash the sections well in a petri dish of water containing 10 drops of ammonia. Carry through two dishes of pure water.

(2) Place in a 12 c.c. pyrex glass cup containing a 2 per cent solution of silver nitrate, to which 3-4 drops of pyridine have been added.

(3) Heat gently for ten minutes at 45°C., when the gray matter will become yellow.

(4) Wash very quickly and place in a similar pyrex dish containing a 5 per cent solution of silver carbonate ("sosa") with 3-4 drops of pyridine.

(5) Heat gently for ten minutes at 45°C., after which the tissue will take on a tobacco color.

(6) Wash for fifteen seconds.

(7) Reduce in ten per cent formol.

(8) Tone in yellow gold chloride solution, 1:500, in the cold for five to ten minutes. Reinforce by heating the toning bath gently for one minute. Wash quickly.

<sup>3</sup> Rio-Hortega, Pio del: Una sencilla técnica para teñir rápidamente neurofibrillas y fibras nerviosas. *Bol. de la Soc. españ. de Histor. Natur.*, 21: 364-371, 1921.

(9) Fix in a 5 per cent solution of "hypo," dehydrate in 96 per cent alcohol, creosote, blot dry, balsam, etc.

For material fixed in F.A.B., or for refractory material fixed in formol for several months:

(1) Cut sections into water containing 10 drops of ammonia.

(2) Heat in a pyrex cup containing 10 c.c. of 96 per cent alcohol and 10 drops of ammonia, for ten minutes at 45°C.

(3) Wash well in three dishes of water.

Employ the double impregnation technique as above, reducing in 1 per cent formol without washing after the silver carbonate treatment.

Basic fuchsin or Roux magenta may be used to counterstain.

*Discussion:* For *degenerated boutons* or for *pathological* changes in neurofibrils omit the reduction in formol, passing from the silver carbonate bath (extending for twelve to fifteen minutes in this case) through two very thorough washings in water to the gold toning bath. Intracellular neurofibrils are best stained by placing frozen sections in 2 per cent silver nitrate for twenty-four hours, followed by heating for ten minutes at 45°C., and continuing as usual.

To prevent connective tissue staining in certain sections, it is necessary to employ 5-10 per cent silver nitrate in the first bath. No pyridine can be added in such cases because of the precipitate it produces. Follow with a 5-10 per cent solution of silver carbonate, without pyridine. An important factor in staining the finest neurofibrils is the reduction process. A trial reduction should be made using 1 per cent or 10 per cent formol as required, then without washing in water, or after a rapid immersion in 70 per cent alcohol, or after washing in water containing a few drops of pyridine.

The addition of 3-4 drops of ammonia to the "hypo" bath accelerates fixation and makes the sections more flexible. Overheating causes the connective tissue to fragment and a black precipitate covers the section.

For *sympathetic ganglia* and *cerebral cortex* a combination of the ammonia-alcohol treatment of the frozen sections, and the silver nitrate 2 per cent solution for twenty-four hours as above, give the best results.

#### IV. Silver Nitrate Method for Frozen Sections

Using Cajal's modification for frozen sections (p. 461) with minor changes, it has been possible to demonstrate bouton endings in the cerebral cortex, cerebellum, and superior cervical sympathetic ganglion.

(1) Blocks of tissue, fixed for at least one week in 20 per cent formol, are cut on the freezing microtome at 12-15μ.

(2) Wash the sections well in water and place in a solution of 12 c.c. of 2

per cent silver nitrate, 6 c.c. of 96 per cent alcohol, and 5-10 drops of pure pyridine, for six to ten hours at 37°C., or twelve to twenty-four hours in the cold. The latter is desirable for the finest detail. Silver nitrate solution up to 5 per cent has been employed successfully in the cold.

(3) Transfer sections to a dish of 98 per cent alcohol where they may remain up to three minutes, depending on the depth of the silver impregnation.

(4) Reduce for three minutes in a solution of 0.3 gm. of hydroquinone, 70 c.c. of water, 20 c.c. of formol, and 15 c.c. pure acetone.

(5) Wash in water and tone in yellow gold chloride solution, 1:500, for five to ten minutes.

(6) Fix in 5 per cent "hypo," wash, mount, dehydrate, balsam, etc.

## V. Block Silver Methods

The following methods are employed in De Castro's<sup>4</sup> laboratory for demonstrating bouton endings and motor or sensory terminations. The usual block impregnation with silver nitrate solution underlies all these techniques, which vary chiefly in the fixatives used.

### *Formula 118.*

(1) Pieces of tissue 4-6 mm. thick are fixed in a solution containing 2-4 gm. of either chloral hydrate, ethyl urethane, or Roche's somnifene, in 50 c.c. of water and 50 c.c. of 95 per cent alcohol. To this solution may be added 1-2 c.c. of 40 per cent nitric acid, depending upon the organ to be stained. This addition prevents the connective tissue staining, and gives a clear impregnation. For terminations in skin, glands, arteries or muscles, the nitric acid should not exceed 1 per cent, while for sympathetic ganglia and cerebral cortex it should be omitted altogether.

(2) After twenty-four hours fixation wash the tissue in running water for six to twelve hours. This time may be reduced to one hour in the case of tissues fixed without the use of nitric acid.

(3) Place the blocks for eight to twelve hours in 50 c.c. of 95-98 per cent alcohol with 2-3 drops of ammonia. One drop suffices if no nitric acid was used.

(4) Wash rapidly to remove surface alcohol.

(5) Incubate the blocks in a 1.5 per cent solution of silver nitrate at 37°C. for five to seven days.

(6) Wash well in water and reduce for twenty-four to forty-eight hours in pyroformol.

(7) Dehydrate blocks, imbed in paraffin, section at 12-15 $\mu$ , and mount in balsam.

Toning in yellow gold chloride solution 1:500 can be carried out. As counterstains, hematoxylin, carmine or basic aniline dyes are recommended.

<sup>4</sup> Cajal, S. Ramón y, and De Castro, F. *Elementos de Técnica Micrográfica del Sistema Nervioso*. Madrid, Tipografía Artística, 1933.

**Formula 119.**

This pyridine fixation method is applicable to embryos, adult non-medullated fibers, the calices of Held, peripheral nerve terminations, and nerves in decalcified tissues. It has been found most useful in studying regenerating nerves.

- (1) Fix tissues in 50 per cent pyridine for twenty-four hours.
- (2) Wash specimens for twelve to twenty-four hours in running water.
- (3) Immerse in 96 per cent alcohol for eight to twelve hours.
- (4) Wash rapidly in water, and impregnate with 1.5 per cent silver nitrate solution at 37°C. for five to seven days.
- (5) Reduce in pyroformol as above.

**Formula 120.**

Chloral hydrate-alcohol-pyridine fixation has produced good results in the *cerebellum*, where the non-medullated fibers have been clearly and darkly impregnated. Both *motor and sensory terminals* of the periphery have been stained also.

- (1) Fix tissues in small blocks for twenty-four to forty-eight hours in a bath containing 3.5 gm. chloral hydrate in 50 c.c. of 96 per cent alcohol and 15-20 c.c. of pure pyridine. The addition of 1 c.c. of water to the above sometimes improves the definition in peripheral nerve terminals.
- (2) Wash in running water for twelve hours.
- (3) Immerse in 95-98 per cent alcohol for twelve hours.
- (4) Wash rapidly in water and incubate in 1.5 per cent silver nitrate solution for three days.
- (5) Reduce the blocks in pyroformol.

**VI. Solutions**

*Five per cent silver carbonate ("sosa"):*

Solution of 10 per cent silver nitrate .....	67 c.c.
Solution of sodium carbonate 5 per cent.....	267 c.c.
Water .....	to 1000 c.c.

Add ammonia drop by drop while shaking the solution until the precipitate just dissolves. Filter and store in a dark bottle.

*Ten per cent silver carbonate ("fuerta"):*

Prepared as above, but water is added up to 500 c.c. only.

*Pyroformol reducer:*

Pyrogalllic acid (Scherring's resublimed).....	1 gm.
Formol .....	5-10 c.c.
Water .....	70-100 c.c.

(Hydroquinone is not recommended as a reducer.)



FIG. 1.



FIG. 2.

FIG. 1. Boutons terminaux after five days' degeneration, on anterior horn cell of spinal cord of cat. Method Cajal 6a. (Chloral hydrate, p. 458.)

FIG. 2. Boutons terminaux as in Figure 1 after twenty-four hours' degeneration.



FIG. 3. Circular "bouton de passage" connected to an oval "bouton terminal" on a dendrite in cat's cerebral cortex. Double impregnation technique.



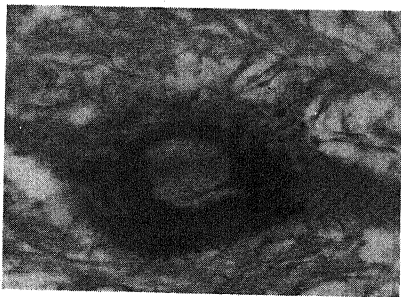


FIG. 4.

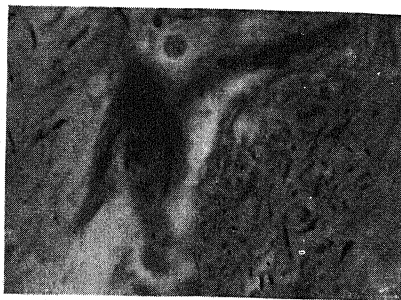


FIG. 5.

FIG. 4. Normal boutons terminaux on anterior horn cells in spinal cord of cat. Cajal 6a technique.

FIG. 5. Terminal bouton on dendrite of cell in cerebral cortex of cat. Double impregnation technique.

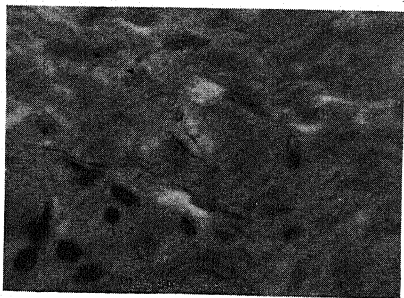


FIG. 6.



FIG. 7.

FIG. 6. Boutons on the surface of a cell in the superior cervical sympathetic ganglion. Double impregnation.

FIG. 7. Showing surface distribution of boutons terminaux on anterior horn cells of cat's spinal cord. Cajal 6a.



FIG. 8.

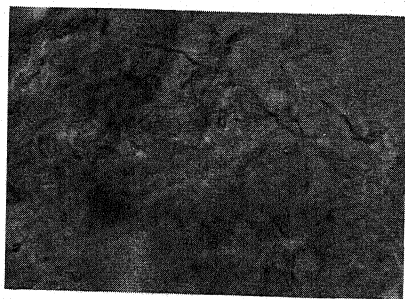


FIG. 9.

FIG. 8. Degenerating bouton terminal on cell body in cerebral cortex of cat. Double impregnation.

FIG. 9. Degenerating boutons in superior cervical sympathetic ganglion of cat three days after cutting preganglionic fibers. Cajal's method for frozen sections.

# NEUROGLIA AND MICROGLIA (THE METALLIC METHODS)

WILDER PENFIELD AND WILLIAM CONE

INTRODUCTION 489. Classification of interstitial cells of central nervous system 489. An outline for routine study of interstitial cells 495. NEUROGLIA ASTROCYTES (MACROGLIA) 496. Cajal's gold chloride sublimate method 497. General observations on silver staining 503. Del Rio-Hortega's silver carbonate method for astrocytes 505. Achúcarro's tannin-silver method 509. OLIGODENDROGLIA 510. Del Rio-Hortega's silver carbonate method for oligodendroglia 510. MICROGLIA 512. Del Rio-Hortega's silver carbonate method for microglia 512. SPECIAL METHODS 515. SOLUTIONS 519.

## A. INTRODUCTION

A word of description may be given here because of the recent additions to our knowledge of the interstitial cells.<sup>1</sup> Like most real advances this added knowledge has resulted in simplification of some problems which formerly seemed complex. The origin of compound granular corpuscles, rod cells, ameboid glia, the so-called preameboid cells, etc., is now clear, making the pathology of the nervous system a much less bewildering field of endeavor than before. The investigation of tumors arising in the brain has also received fresh impetus.<sup>2</sup>

## I. Classification of the Interstitial Cells of the Central Nervous System

	<i>Normal Forms</i>	<i>Pathological Forms</i>
Neuroglia	<ul style="list-style-type: none"> <li>Astrocytes <ul style="list-style-type: none"> <li>a. Protoplasmic (gray matter)</li> <li>b. Fibrous (white matter)</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>Ameboid glia</li> <li>Fibrous gliosis</li> <li>Giant glia</li> </ul>
Microglia	<ul style="list-style-type: none"> <li>Oligodendroglia <ul style="list-style-type: none"> <li>a. Perineuronal satellites</li> <li>b. Interfascicular</li> </ul> </li> <li>Ubiquitous</li> </ul>	<ul style="list-style-type: none"> <li>Acute swelling</li> <li>Satellitosis</li> <li>Rod cells</li> <li>Compound granular corpuscles.</li> <li>(Gitterzellen)</li> </ul>

1. **Astrocytes** (classical neuroglia, macroglia). The most complete description of these cells is that made by Cajal in 1913<sup>3</sup> after devising the

<sup>1</sup> Penfield, W. In: Cowdry, E. V. (ed.). *Special Cytology*. N. Y., 1928.

<sup>2</sup> Bailey, P., and Cushing, H. *Tumors of the Glioma Group*, Phila., 1926.

Penfield, W. *Nelson's Loose-Leaf Surgery*, N. Y., 1927.

<sup>3</sup> Cajal, S. Ramón y. *Trab. d. Lab. d. Invest. Biolog. d. I. Univ. d. Madrid*, 2: 255, 1913.

gold chloride method. The astrocytes are star-shaped cells whose expansions radiate through the nervous tissue in all directions. They all possess one or more perivascular expansions which are applied to the sur-



FIG. 1. Protoplasmic astrocytes.

face of small vessels. They possess a granular cytoplasm which contains gliosomes, small granules said to be secretion granules.

Protoplasmic astrocytes (Fig. 1) are found in the gray matter. They are in general somewhat smaller than fibrous astrocytes, contain more gliosomes, and have more irregular expansions. They normally have no fibers but develop them under numerous pathological conditions.

Fibrous astrocytes (Fig. 2) contain long slender fibers of Ranvier-Weigert which pass through the cell body and out into the expansions. Under normal circumstances the fibers are not divorced from the cell and gliosomes can be seen to accompany them. Selective fiber stains such as Weigert's may give an erroneous appearance of fiber divorcement.

Pathological changes occur rapidly in astrocytes, particularly the

ameboid change of Alzheimer (clasmatodendrosis of Cajal), (Fig. 3). This may appear as an acute change in various toxic conditions of the nervous system, as an agonal change and also as a post-mortem change.



FIG. 2. Fibrous astrocyte.

It is found earlier in the white matter and often it affects one cell and spares the next. In staining human material the characteristics of this change must be kept constantly in mind. The fragmented expansions (Fullkörperchen) seen in Figure 3 may otherwise be taken for artifacts.

2. **Oligodendroglia** (oligoglia, Robertson's mesoglia). These cells were first described by Robertson in 1900.<sup>4</sup> He used a platinum method which proved so unreliable as not to warrant description in these pages. Del Rio-Hortega in 1921 provided a better method and a full description of these cells.

Oligodendroglia cells are by far the most numerous of the supporting cells in the nervous system. They occur in rows in the white matter (Fig. 4) where their expansions interlace about the medullary tubes. They thus hold a position relative to nerve-fibers, comparable to that of the sheath of Schwann cells in the peripheral nervous system. They are also present about nerve-cells (Fig. 5) appearing as "satellites" espe-

<sup>4</sup> Robertson, W. *Scot. Med. & Surg. J.*, January, 1899. *J. Med. Sc.*, October, p. 724, 1900.

cially in the neighborhood of the axon hillock. These cells contain gliosomes similar to astrocytes. They do not possess perivascular attachments and form no fibers. They are much smaller than most astrocytes. Their

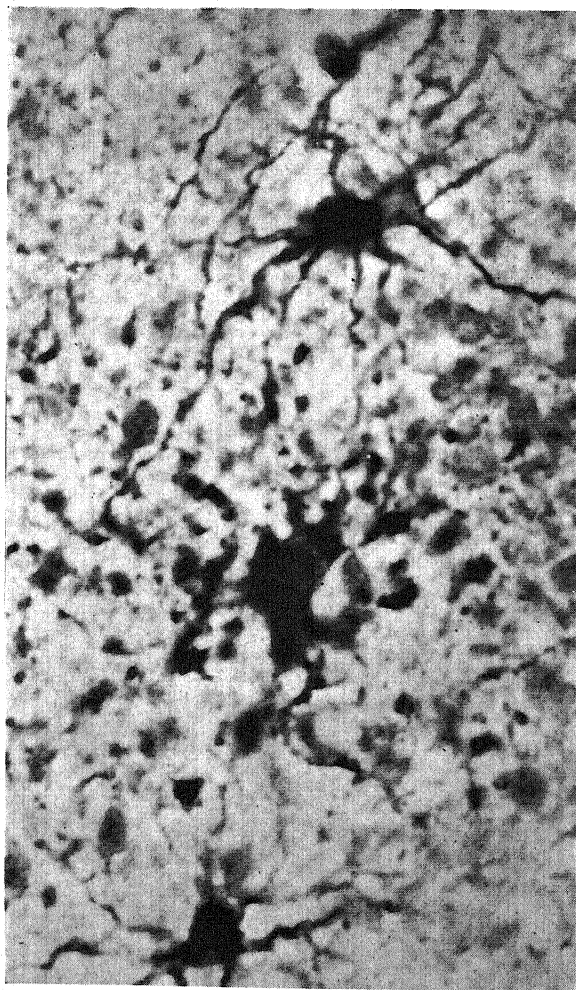


FIG. 3. Clasmotodendrosis of fibrous astrocyte. Also unaltered cell.

small rounded nuclei are seen in ordinary stains without cytoplasm.

Normal oligodendroglia are not ordinarily seen in human material unless death is sudden and the autopsy early. Material obtained at operation and following accidental death contains oligodendroglia similar to that of sacrificed animals. If death is preceded by coma or deep stupor



FIG. 4. Oligodendroglia of the white matter (interfascicular).



FIG. 5. Perineuronal oligodendroglia (satellite).

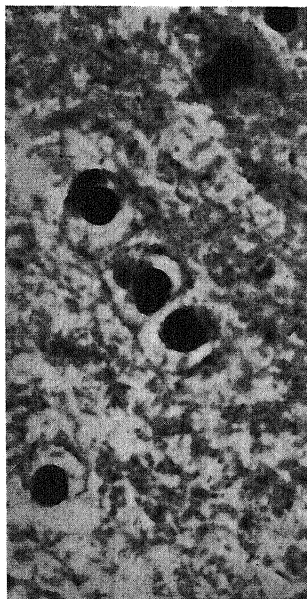


FIG. 6. Acute swelling of intrafascicular oligodendroglia.

there will be found acute swelling of oligodendroglia (preameboid glia),<sup>5</sup> (see Figs. 6 and 7). If there is a toxic or infectious cerebral condition this swelling is extreme and may go on to cell destruction before death.

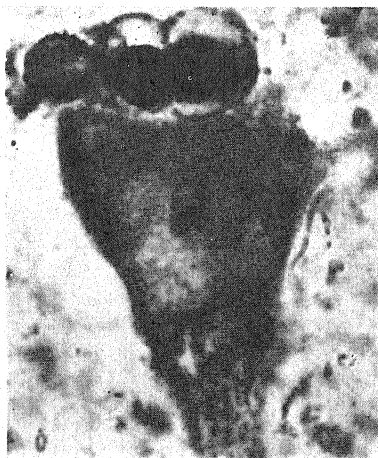


FIG. 7. Acute swelling of perineuronal oligodendroglia.

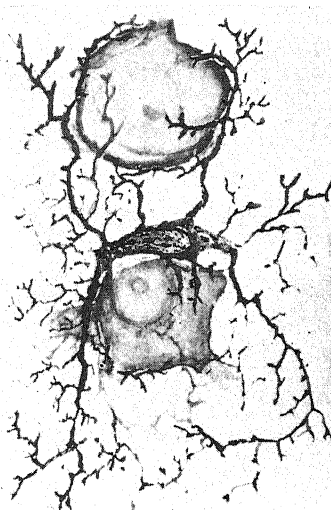


FIG. 8. Microglia.

This acute swelling occurs also as an autolytic process. It is a more sensitive change than clasmotodendrosis and occurs earlier but as the result of the same influences. In certain conditions also there is an increase in the number of perineuronal oligodendroglia amounting to a "satellitosis."

**3. Microglia** (mesoglia, Hortega cells). These cells were first described under normal conditions by Del Rio-Hortega in 1919.<sup>6</sup> In their pathological forms they have long been recognized as rod cells and compound granular corpuscles. These cells are small in comparison with astrocytes. They have irregular protoplasmic expansions (Fig. 8) with stubby, spinelike projections. The nucleus is usually elongated or triangular and contains a heavier chromatin net than that found in neuroglia. There are no gliosomes and no fibers. The cells are not sensitive to the same toxic, agonal and autolytic influences as neuroglia.

The development of these cells is quite different from that of neuroglia, both forms of which develop from ectodermal spongioblasts. Microglia seems to develop from the pia mater and possibly vascular adventitia and is therefore probably of mesodermal origin. These cells correspond

<sup>5</sup> Penfield, W., and Cone, W. *Arch. Neurol. & Psychiat.*, 16: 131, 1926.

<sup>6</sup> Del Rio-Hortega, P. *Boletín d. l. Soc. Esp. d. Biología*, 9: 68, 1919.

to macrophages elsewhere in the body and may be grouped in the reticulo-endothelial system. The methods given here for microglia will stain the other elements of that so-called system. Microglia cells react



FIG. 9. Intermediate form of microglia near brain wound.



FIG. 10. Compound granular corpuscle.

promptly to destructive processes<sup>7</sup> passing rapidly through transitional forms (Fig. 9) to become phagocytic "Gitterzellen" (Fig. 10).

The methods to be described may be divided into three groups according to the cells stained: (a) astrocytes, (b) oligodendroglia, (c) microglia. By the first group all types of astrocytes may be stained but small modifications of these methods may demonstrate the protoplasmic type better than the fibrous, and vice versa. In general, the pathological forms of all three types of cells are more easily demonstrated by the metallic methods than are the normal forms.

Oligodendroglia may be impregnated in the gray and the white matter with equal facility. Microglia cells are studied more easily in the gray matter where they are much more numerous. When oligodendroglia is well stained microglia is usually stained also. But either cell group may be stained with exclusive selectivity. At times when using silver carbonate, astrocytes may be incompletely stained along with the two groups of smaller cells.

## II. An Outline for the Routine Study of the Interstitial Cells

It is necessary in most pathological laboratories to have a routine procedure for use in the study of the brain and spinal cord. When certain aspects are of importance, as in a particular specialized research problem, the material may be treated with a view to the exclusive demonstration

<sup>7</sup> Penfield, W. *Am. J. Path.*, 1: 77, 1925.



of one cell group as described in the methods below. As a rule, however, it is necessary to compromise to some extent so as to use various methods with the best average result. The following outline may be found useful for that purpose:

**Outline.** Remove the brain and cord as soon after death as possible. It is preferable to inject the carotid artery early with 10 per cent formol to secure the best possible cell preservation. If this is not feasible excellent and immediate fixation of the whole brain may be accomplished by injecting the basilar artery of the brain after the cut branches of the circle of Willis have been ligated. This injection does not seem to prevent good results even with Nissl's or Cajal's methods if blocks are taken for these procedures immediately after the removal of the brain, and the preservation of neuroglia is thereby greatly improved.

Remove some blocks from the brain at once and place them in alcohol for Nissl's stains. (For a thorough survey of the interstitial cells of the various areas Orton's method<sup>8</sup> of taking blocks is orderly and very useful.) Place other blocks in formalin-ammonium-bromide solution in the 38°C. incubator for Cajal's gold chloride method (page 519). These blocks are cut and stained at the end of forty-eight hours and if desired some of the sections may be stained for oligodendroglia by Hortega's method (1) at the same time. Place the brain and cord in 10 per cent formalin. The removal of these preliminary blocks may be omitted if it is desired to keep the brain intact until it can be photographed. But results for these two methods are then not so good.

On the fifth day make gross photographs and cut the brain. Blocks are then taken for any of the various methods as outlined in Chapter I. Blocks are also cut on the freezing microtome and the sections stained by each of the following methods: Globus' modification of Cajal's gold chloride method (page 500); Penfield's second modification of Hortega's silver carbonate method for both microglia and oligodendroglia (page 511); Bielschowsky's or preferably Hortega's method for neurofibrils and Hortega's or Perdrau's method for connective tissue. If the gold chloride method for astrocytes should have proved unsatisfactory the method of Hortega can be tried on the sections from the same block or on other blocks after longer fixation.

### B. NEUROGLIA ASTROCYTES (MACROGLIA)

The bichromate silver method of Golgi<sup>9</sup> was long the best staining procedure for classical neuroglia and because of the appearance of these

<sup>8</sup> Orton, S. T. *Am. J. Insan.*, 69: 429, 1912.

<sup>9</sup> Golgi, C. *Opera Omnia*. 3 vols. Milan, 1903.

Lee, A. B. *The Microtome's Vade-mecum*. Ed. 9, London, 1928.

cells they were considered as independent structural units. The splendid method of Weigert then came into use and showed neuroglia fibrils with such selectivity that they came to be considered as extra-cellular structures.

There followed many other methods, some of which added important details, while others stained neuroglia so indistinctly that these cells seemed to merge in with a vague diffuse syncytial network. There are many important methods which cannot be described here, such as Weigert's,<sup>10</sup> Benda's,<sup>11</sup> Alzheimer's,<sup>12</sup> Mallory's phosphotungstic hematoxylin method (p. 427), Anglade and Morel's Victoria blue method, Lhermitte's,<sup>13</sup> Bailey's,<sup>14</sup> et al.

The method of Achúcarro provided in 1911 a very valuable means of studying astrocytes. But the advent of Cajal's gold chloride method in 1913 demonstrated these cells both protoplasmic and fibrous with unequalled clarity and selectivity. The cells could be seen as cell entities stained completely with gliosomes, glia fibrils, perivascular feet, etc. The method of Del Rio-Hortega provided in 1917 another photographic procedure which when successful cannot be excelled and which is to be preferred to the gold method if the tissue has been long in formalin.

## I. Cajal's Gold Chloride Sublimate Method for Neuroglia Astrocytes<sup>15</sup> (G. C. S.)

### 1. The Method.

(1) *Harden.* Blocks of tissue not over 5 mm. in thickness should be removed from the central nervous system as soon as possible after death and placed in formalin-ammonium-bromide solution (solution c under "Solutions" p. 519). Allow blocks to harden in this solution at room temperature for four to twenty-

<sup>10</sup> Weigert, C. *Beitrage zur Kenntniss der normalen menschlichen Neuroglia.* Frankfurt, 1895.

<sup>11</sup> Benda. See chapter on "Neurogliafarbung." *Enzykl. mik. Technik*, 1910, vol. 2.

<sup>12</sup> Alzheimer, A. *Histolog. u. histopath. Arb. Nissl-Alzheimer*, 3: 401, 1910.

Spielmeyer, W. *Technik der mikroskopischen Untersuchung des Nervensystems.* Berlin, 1914.

<sup>13</sup> Roussy, G., and Lhermitte, J. *Les techniques anatomo-pathologiques du systeme nerveux.* Paris, 1914.

<sup>14</sup> Bailey, P. J. *Med. Research*, 44: 73, 1923.

<sup>15</sup> Cajal's successive descriptions of this method provide advice and warnings concerning the method but make no fundamental alterations in original procedure. If the results are not uniformly good, reference may be made to the original descriptions by this master of technique, but as much as possible of his advice is included here. Where there is a difference the conclusion last published is given.

Cajal, S. Ramón y. *Trab. d. Lab. d. Invest. Biolog. d. Univ. d. Madrid*, 2: 219, 1913; 14: 155, 1916; 18: 129, 1920.

five days. In general, protoplasmic astrocytes stain better with a short fixation time and fibrous astrocytes after longer fixation.

(2) *Cut.* Sections are cut on the freezing microtome at about  $25\mu$  and placed directly in distilled water to which a few drops of formalin have been added to prevent swelling of sections.

(3) *Wash.* Pass the sections rapidly through two changes of distilled water in Petri dishes and place them in the gold bath.

(4) *Stain.* Place sections in a flat dish which is filled to the depth of about 1 cm. with gold chloride sublimate solution (solution d). Flatten out each section on the bottom of the dish so that it is not folded or overlapped by other sections. Place the dish in the dark, preferably in an oven at a temperature of  $22^{\circ}\text{C.}$ , for human cerebrum where four to six hours will be required (see technical discussion). When sections begin to turn an intense purple remove them.

(5) *Fix.* Transfer sections from gold bath to Cajal's fixing bath (solution e 519). Leave them here six to ten minutes.

(6) *Wash.* Wash sections in 50 per cent alcohol and mount on glass slip.

(7) *Dehydrate.* Blot section on slip and dehydrate with absolute alcohol.

(8) *Clear.* Oil of origanum and xylol.

(9) *Mount.* Canada balsam.

**2. Discussion of the Steps of the Method.** (1) *Hardening.* Ammonium iodide may be substituted for the bromide, this combination being better than formalin alone. Also, according to Cajal 25 per cent of methyl alcohol may be used in the fixative, or 2 per cent of acetanide, also carbamide nitrate in the same strength. This last substance prevents granular appearance but lessens the energy of the impregnation. If it is desired to study fibers of Ranvier-Weigert, this last salt may be used to good advantage.

In case the tissue has been hardened in formalin without ammonium bromide, successful staining may at times be obtained; indeed, this was the fixative first used by Cajal. We have occasionally succeeded by placing old formalin sections in formalin-ammonium-bromide for a time and proceeding as usual.

The optimum duration of hardening for the nervous system of newborn mammals was found by de Castro<sup>16</sup> to be three to four days in formalin-ammonium-bromide. For the human olfactory lobe<sup>17</sup> he secured good results by substituting urea nitrate for ammonium bromide in hardening as follows: formol, 15 c.c.; water, 100 c.c.; urea nitrate, 1 to 2 gm. He also successfully employed for this tissue; formol, 15 c.c.; water, 100 c.c.; commercial ammonium carbonate, 2 gm.

(2) *Sections.* In the opinion of Cajal the most favorable results are obtained with thick sections which also permit one to follow the sprawl-

<sup>16</sup> Castro, F. de. *Trab. d. Lab. d. Invest. Biolog. d. l. Univ. d. Madrid*, 14: 83, 1916.

<sup>17</sup> *Ibid.*, 18: 1, 1920.

ing astrocyte expansions through their full extent. In our hands, sections cut at about  $15\mu$  are preferable for staining and make photography easier.

Cajal has pointed out that one weakness in the method is that the superficial tissue is apt to stain less well than the deeper tissue, due to the fact that the more energetic action of formalin at the surface carries the superficial tissue too far, destroying the aurophilic properties of the astrocytes by the time the deeper cells are in a condition suitable for staining. This may be avoided by leaving the pia intact or covering with a layer of blood or other substance.

(3) *Staining.* The importance of the temperature of the gold sublimate bath must not be overlooked. Cajal concluded that  $18$  to  $22^{\circ}\text{C}$ . is the proper temperature for the human cerebrum. At such temperatures the time consumed is usually four to eight hours. For mammals low in the phylogenetic scale and for birds, reptiles and fish the optimum temperature is  $25$  to  $35^{\circ}\text{C}$ . For cerebellum, bulb and spinal cord, a somewhat higher temperature of gold bath is desirable according to Cajal, i.e.,  $25$  to  $28^{\circ}\text{C}$ . In the pineal gland, Del Rio-Hortega was able to stain neuroglia only if a temperature of  $27$  to  $30^{\circ}\text{C}$ . was used. De Castro prefers for the olfactory bulb of cats, dogs and rabbits the temperature of  $24$  to  $27^{\circ}\text{C}$ . and for the human olfactory lobe  $35$  to  $40^{\circ}\text{C}$ . which accomplishes the reaction in less than an hour. For the nervous system of newborn mammals he advises  $24$  to  $26^{\circ}\text{C}$ .

Although Cajal advises placing the gold bath in the dark, we have not observed any detriment to the process when it is accomplished in daylight. When the sections are becoming purple it is well to remove and mount a single section to see if the reaction is complete. The astrocytes should stand out like black stars even when examined wet under the microscope.

If the room is cold the sublimate may be doubled, or the gold doubled and the sublimate tripled. Brevity and energy of reaction may also be obtained by adding 2 to 3 drops of a 1 to 1000 solution of erythrosin.

(4) *Fixing.* As first used by Cajal the sections were not fixed but he found the preservation not perfect without fixation. It is our custom to wash before passing the sections into the fixative as is done by Del Rio-Hortega. We have also substituted ordinary photographic hyposulphite of soda, 5 per cent, for the more complicated fixing bath of Cajal with better results in our hands.

(5) *Dehydration and Mounting.* It is our custom to dehydrate clear and mount after the fashion of Del Rio-Hortega in his silver methods, using carbol-xylol-creosote to clear after 95 per cent alcohol followed by balsam. Some of our preparations thus mounted are perfectly preserved for over three years.

a. *Modification of Globus.* Globus<sup>18</sup> has provided a method of rendering tissue hardened in formalin available for staining with gold, which to quote from his description is as follows:

- (1) Prepare frozen sections of formaldehyde-fixed material at a thickness of from 15 to 30 $\mu$ .
- (2) Wash quickly in several changes of distilled water.
- (3) Place in a 10 per cent solution of strong ammonia water for twenty-four hours at room temperature, or for shorter periods in an incubator.
- (4) Carry rapidly through two changes of distilled water.
- (5) Place in a 10 per cent solution of (pure, 41 per cent) hydrobromic acid and let it remain there for two to four hours.
- (6) Wash quickly in two changes of distilled water, to which a few drops of ammonia water are added and place section in gold chloride solution as usual.

In our experience, this works out very well provided the tissue has not been too long in formalin.

3. **Underlying Principles of the Gold Sublimate Method.** The chemical formulas involved in the reaction are unknown. As has been said, results which are at times quite favorable may be secured after fixation in formalin alone. The addition to the fixative of ammonium bromide, ammonium iodide or potassium iodide improves the vigor of the eventual impregnation and lessens the tendency to granular staining.

The presence of the corrosive sublimate in the gold bath seems to be indispensable to a successful outcome. Gold chloride alone will color the tissue diffusely. The addition of mercuric chloride seems to render the astrocytes aurophilic and to accelerate the deposit in these cells of metallic gold. It is possible that mercury, which is capable of going into solution with the gold, withdraws the latter metal from all the cells except astrocytes. The action of the final fixing bath is only to preserve the preparation.

In the protoplasmic astrocytes, as pointed out by Cajal, the staining affinity of the terminal expansions seems to be a little different from that of the cytoplasm of the cell body, for when these expansions are energetically impregnated the cell body and nucleus are more faintly stained, and vice versa.

This is not true of the fibrous astrocytes but there is definite difference in reaction between the fibrous and the protoplasmic astrocytes. In general the latter cells in the gray matter reach their optimum for staining after two or three days of hardening, while the fibrous astrocytes of the white matter stain best at a later period and continue to be aurophilic after the protoplasmic cells have become refractory to gold.

The action of the formalin, if allowed to continue, eventually renders all the astrocytes refractory to gold.

<sup>18</sup> Globus, *C. Arch. Neurol. & Psychiat.*, 18: 263, 1927.

4. **Results.** With an insignificant amount of labor the astrocytes (classical neuroglia) are stained in an exquisitely selective manner. The astrocytes of the white matter are shown completely with their fibers and particularly their perivascular feet. If the staining is not intense the fibers can be seen to pass through the cytoplasm of the cell body and out into the expansions. With more diffuse impregnation the individual fibers are lost sight of and only the shape and length of the expansion indicate that it is fibrous. It must be remembered that astrocytes often undergo an agonal or post-mortem change (clasmotodendrosis) consisting of swelling and fragmentation of the expansions.<sup>19</sup> Autopsy material must therefore be obtained as quickly as possible after death.

But it is in the staining of the protoplasmic astrocytes that the gold-sublimate method excels all previous methods. The protoplasm can be followed out to terminal expansions and the perivascular feet are easily demonstrated. Gliosomes, small, smooth, oval or round granules, may be at times stained with great distinctness.

Neuron bodies may be made out very indistinctly but axons and myelin sheaths are completely unstained. Oligodendroglia cells are unstained, although a halo is seen about these nuclei. Likewise microglia (mesoglia of Hortege) are unstained except for their elongated nuclei. The lepto- and pachymeninges are invisible, as are also the blood vessels. Capillaries may be outlined by well-stained perivascular glia feet.

The method works particularly well on human material. Of laboratory animals, dogs and cats give particularly clear pictures. Rabbits in our experience give only fair results. Del Rio-Hortega's method is preferable for these last animals. Splendid impregnations will be secured with newborn animals, astroblasts being well demonstrated. When the method is used on gliomas only those cells are stained which have progressed to the astrocyte stage. The more embryonic forms do not stain at all. It is useful therefore as an indicator of the degree of differentiation of these neoplasms. The giant cells in spongioblastoma multiforme may stain and the cells of the astrocytomas should stain well, although in our hands, so far, these tumors are sometimes refractory to gold, while the phosphotungstic acid method of Mallory may succeed better. The astrocytes that appear in ependymomas stain well, while none of the cells of medullo-blastomas are impregnated.

The procedure can be carried out in a routine standard manner if the proper chemicals are obtained, if the glassware is scrupulously clean and if attention is paid to the above advice with regard to time of fixation.

One difficulty with the method is that occasionally the astrocytes,

<sup>19</sup> Penfield, W., and Cone, W. J. *f. Psychol. u. Neurol.*, 34: 204, 1926.

though selectively stained, are quite granular. This may be due, among other causes, to leaving sections too long in the gold bath, too long hardening, or overheating.

The best results will be obtained with the use of formalin-ammonium-bromide as fixative. Good results may be obtained, however, with the use of the Globus modification after formalin fixation, provided the time of formalinization is not too great. In our experience, with an occasional exception, material which has hardened in formalin longer than six weeks gives only fair results with gold chloride, whatever modification be employed.

**5. Routine Use of the Method.** The routine procedure which we have gradually come to use during the past four years will be outlined below. We do not propose it as a new modification. In addition to the personal alterations there are some details in it derived from Del Rio-Hortega and there is an important step borrowed from Globus.

(1) *Harden* thin blocks in formalin-ammonium-bromide solution one day in the incubator at 38°C.

(2) *Cut* sections on the freezing microtome at 15 $\mu$  thickness and place directly in 1 per cent formalin.

or

(1) *Harden* the whole brain in 10 per cent formalin for a week at room temperature, preceded, if possible, by injection of formalin at the earliest possible moment after death. In experimental animals the injection is made while the animal is under ether anesthesia. If a pressure of about 2½ meters is used it is not necessary to wash out the vessels with any other solution. When the brain has been thus injected the second step should be taken early, even before twenty-four hours.

(2) *Cut* as above, wash and follow the Globus procedure by placing sections in a closely covered vessel containing distilled water to which is added about 1 drop of strong ammonium hydroxide for every cubic centimeter of water. Leave in this overnight. Then wash sections quickly and place them in 10 per cent hydrobromic acid for one hour in the incubator at 38°C.

(3) *Wash* rapidly in two changes of distilled water.

(4) *Impregnate*. Place not over six sections in a flat porcelain dish provided with porcelain cover and containing about 25 c.c. of freshly prepared gold sublimate solution. This dish is used for no other purpose and the glassware for preparing gold bath is used for nothing else as a further security against contamination.

The bath is prepared as described under Solution d, except that the amount of mercury bichloride is doubled, i.e., mercury bichloride 1 gm.; water 50 c.c.; and gold chloride (1 per cent) 10 c.c.

The sections are laid flat on the bottom of the vessel and kept in the dark.

When the purple begins to appear, one section is removed and examined wet and if the astrocytes are seen, it is mounted. The optimum color is usually a reddish purple. A little reddish deposit in the solution is of no consequence, but if a scum begins to form the sections should be removed.

The bath is at room temperature but during the heat of the summer the results are apt to be unsatisfactory.

- (5) *Wash* in distilled water.
- (6) *Fix* the sections in 5 per cent sodium hyposulphite (photographic "hypo").
- (7) *Wash* thoroughly in several changes of water. Extra sections may be placed in 1 per cent formalin and preserved there for long periods.
- (8) *Dehydrate* in 95 per cent alcohol after floating the sections from water on to the slip.
- (9) *Clear* with Hortega's carbol-xytol-creosote mixture (Solution f). Blot as soon as the section is clear and flattened.
- (10) *Mount* in Canada balsam.

## II. General Observations on Silver Staining<sup>20</sup>

In general, each of the silver methods should be carried out with a standardized technique. There are minor differences between them and it may be necessary to try small variations at times to achieve success, but certain rules must be constantly observed. In all the variations of the silver carbonate method, as well as the tannin silver method, the equipment of the table is much the same.

It is best to carry out the silver procedures at the worker's own desk where a microscope can be used to determine the optimum impregnation times, and all solutions are within reach, as in Figure 11. The desk and shelves in this illustration are planned especially for silver staining.

In many of the procedures the sections can be cut, stained, mounted and studied in the same working period. It is only thus that the best results are obtained, for defects in the desired staining result often can be remedied by small variations in the procedure instituted at once.

A bottle of doubly distilled water should be on the table. It stands in the waste jar until it is called into use. The following drop bottles are needed and may conveniently be kept in sockets in a block of wood that may be moved about at will: alcohol 96 per cent; carbol-xytol-creosote; xytol; ammonium hydroxide; pyridine.

For washing sections, Petri dishes which contain 70 c.c. are used. The small glass dishes used for silver, for toning gold and for "hypo" solutions contain 15 c.c.

When it is desired to heat the sections they are placed in a glass of solution upon a tripod and asbestos sheet (Fig. 12) over an alcohol lamp. The glass dish should be filled and covered with a watch glass so that a bubble of air remains

<sup>20</sup> Metallic methods have been well reviewed by Carleton and by Da Fano. Carleton, H. *Histological Technique*. Lond., 1926. Lee, A. *The Microtometist's Vade-mecum*. London, 1928.



inside capable of stirring up the sections when the whole is shaken. The heat should never exceed 50°C., a temperature at which the glass begins to feel disagreeably hot against the finger.

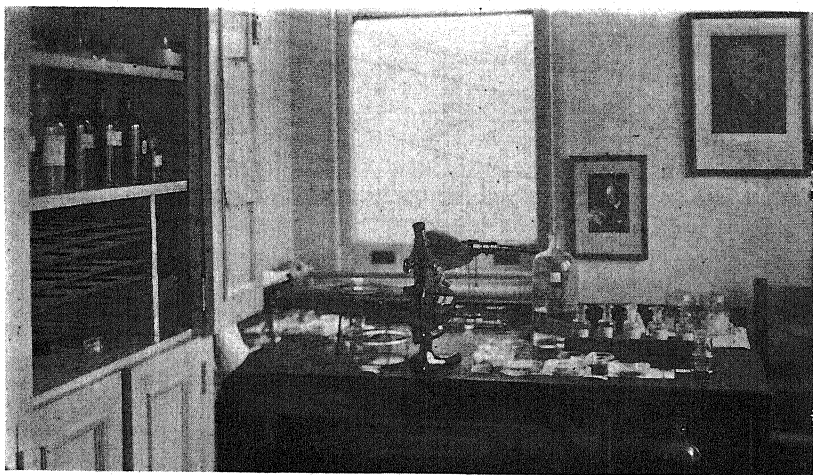


FIG. 11. Desk and cupboard for silver staining.

A powerful artificial light should be used with a blue filter, as many of the sections are quite thick. For examining wet sections a microscopic magnification of about 150 is satisfactory.

The silver of Bielschowsky,<sup>21</sup> though designed for staining neurofibrils, has been used by means of numerous modifications for the staining of neuroglia. Most successful among these is perhaps the method of Achúcarro. This silver solution is prepared by precipitating the silver from silver nitrate solution with sodium hydroxide and redissolving the precipitate with ammonia. The "silver carbonate solution" of Del Rio-Hortega is obtained by precipitating the silver from the silver nitrate solution with sodium carbonate and redissolving that precipitate with ammonia. In the Achúcarro method the sections are passed through a mixture of tannic acid and ammonium hydroxide to prepare them for the silver bath. After the bath the silver in the tissues is reduced in formaldehyde.

In the Hortega methods the sections are prepared in a variety of ways according to which type of cell is to be demonstrated. After passing through the silver bath they, too, are plunged into formaldehyde for reduction.

For these silver procedures ammonium bromide, used to prepare the tissue, improves the staining of astrocytes, just as it does in Cajal's gold

<sup>21</sup> Bielschowsky, M. J. f. *Psychol. u. Neurol.*, 3: 169, 1904.

chloride method. But it is not indispensable for any of them. In the staining of oligodendroglia and microglia by ammoniacal silver carbonate this preliminary use of ammonium bromide in some form seems



FIG. 12. Same desk, one of Hortega's silver carbonate methods in process.

to be indispensable.

Our knowledge is not sufficiently advanced to permit chemical analysis of these metallic methods. As Liesegang<sup>22</sup> concluded, after studying silver staining, a new gel chemistry is required. Formalin in the tissue seems to act as a mordant for the silver. The various accessory mordants used in different modifications serve to render some particular cell group selectively argentophilic, so that when the sections are passed to the reducing formalin silver stains that cell group takes up only the silver. The section after being mordanted and passed through silver has been likened by Liesegang to a photographic plate which had been exposed but not developed. Reduction of the section and development of the plate demonstrate the picture.

### III. Del Rio-Hortega's Silver Carbonate Method for Astrocytes<sup>23</sup> (S. C. A.), (Fig. 13)

#### 1. Method.

*Harden.* The tissue should be cut in blocks less than 1 cm. in thickness and hardened in formalin-ammonium-bromide solution (c). Duration in this fixative should be about twenty to forty days at room temperature for best results with the protoplasmic astrocytes of the gray matter and something over a month's

<sup>22</sup> Liesegang, R. *Kolloidchem. Beihefte*, 3: 1, 1912.

<sup>23</sup> Del Rio-Hortega, P. *Trab. d. Lab. d. Invest. Biol. d. I. Univ. d. Madrid*, 15: 367, 1917.

duration for good results with fibrous astrocytes. It is difficult to give definite dates for hardening as the optimum duration is not constant. The time of hardening may be shortened by placing the tissue in an incubator at 38°C. Good



FIG. 13. Del Rio-Hortega's silver carbonate astrocyte stain. 1. Water. 2. Silver carbonate (lithium), pyridine, alcohol. 3. Water. 4. 1 per cent formol. 5. Water. 6. Gold chloride. 7. "Hypo." 8. Water.

results in both types of astrocytes may then be obtained at the end of two to three weeks.

Results, sometimes quite good results, may be had after hardening in 10 per cent formalin even for very long periods. Staining of protoplasmic astrocytes may be obtained as early as the third day after simple formalin fixation, and the modification of Globus may be used here to bromurate the formalin-fixed tissue (p. 500). Also it is sometimes very helpful to place formalin-fixed sections in Cajal's reinforcer (solution i) for four hours at 38°C. before beginning the staining.

*Section.* Cut sections on the freezing microtome at 15 to 25 $\mu$  and receive in water containing a few drops of ammonia.

(1)<sup>24</sup> *Wash.* Wash well in four changes of water (Figs. 12-1, 2, 3) to get rid of all formol.

(2) *Stain.* Place six to twelve sections in ammoniacal silver carbonate (lithium) (solution g) in a small glass dish which contains about 10 c.c. Add a few drops of pyridine to prevent the formation of a scum on the surface. Cover with a watch glass leaving a bubble and place over the alcohol flame (Fig. 12). Heat to 45 or 50°C. shaking from time to time. Leave sections here, usually three to five minutes, until they become dark amber in color and the liquid takes on a grayish color. (The liquid may become brownish in case the sections have been insufficiently washed.)

If fixation has been short or the first result is unsuccessful remove the sections and heat in a second dish of silver solution similarly prepared except that 12 drops of 96 per cent alcohol are added. Alcohol if desired may be added also to the first silver. Remove sections when an amber color is secured.

It is well to carry one section through to reduction and float onto a slip for microscopical examination to see whether the impregnation is complete or not and whether a second heating in silver is necessary.

(3) *Wash.* Place sections in distilled water. Allow them to fall to the bottom. Then raise them with the glass rod, wash quickly and plunge them into the reducer. Too long washing gives a pallid result.

(4) *Reduce.* One per cent formalin (10 per cent may be used, but is no better in our experience) is used as "reducer." The reduction takes place at once.

<sup>24</sup> A diagrammatic representation of this method is seen in Figure 13. Numbers in the diagram correspond with those of the description.

(5) *Wash.* Wash well.

(6) *Tone.* Place sections in gold chloride solution (h). Leave them here a few minutes till they become gray, then heat, not exceeding 50°C. until the sections become a dark purple.

(7) *Fix.* Pass sections directly to 5 per cent hyposulphite of soda. Leave them here until flexible, about one-half a minute.

(8) *Wash* in water, mount, dehydrate, clear, etc., as for the other methods.

2. **Results.** The general background should be unstained and the astrocytes clearly outlined, the nucleus and pigment granules being clearly stained, and the fibers in the fibrous astrocytes may be distinguished as they pass through the cytoplasm of the cell body. Gliosomes are rarely stained.

When perfectly successful the result is brilliant, the astrocytes are stained completely and the contrasts such that photography is quite easy. On the other hand, the method is variable and less reliable than that of Cajal's gold sublimate method. It is the more reliable of the two methods when the nervous tissue has been long fixed in formol.

For pathological astrocytes, where there has been hypertrophic gliosis, the silver carbonate method gives particularly good results, often staining only the enlarged cells but staining them with the greatest clarity even after ordinary formalin fixation.

If it is desired to study the astrocyte cytoplasm it is better to omit toning the sections and to mount after reduction and washing in water. On the other hand, toning reinforces the glia fibrils and gives better preparations for routine work. If formalin is used for hardening without ammonium bromide the neurofibrils are likely to be stained rather too much.

3. **Modification of Cajal.** *Ammoniacal Silver Oxide Method for Astrocytes.*<sup>25</sup> This is in principle a modification of the foregoing silver carbonate method of Del Rio-Hortega. In our hands it has proved inferior to the silver carbonate method or the gold chloride method. Cajal recommended it for the study of general paresis, as it seems to stain pathological glia well, just as does Hortega's method.

(1) *Harden.* Tissue as fresh as possible placed in formalin-ammonium-bromide for two to twenty-five days.

(2) *Section.* Frozen sections at 25 to 35 $\mu$  are placed in formalin-ammonium-bromide.

(3) *Reinforce.* For more energetic staining (particularly of the cell body) the sections should remain four hours at room temperature (or preferably in the incubator) in the reinforcer (solution i). To stain glia fibrils leave in reinforcer a short time only.

<sup>25</sup> Cajal, S. Ramón y. *Trav. d. Lab. d. Rech. Biol. d. l'Univ. d. Madrid*, 23: 157, 1925.

- (4) *Wash.* Wash rapidly in three changes of distilled water.
- (5) *Stain.* Place in the following ammoniacal silver bath:

Ammoniacal silver.....	10 c.c.
Water.....	10 to 12 c.c.
Pyridine.....	7 to 10 drops

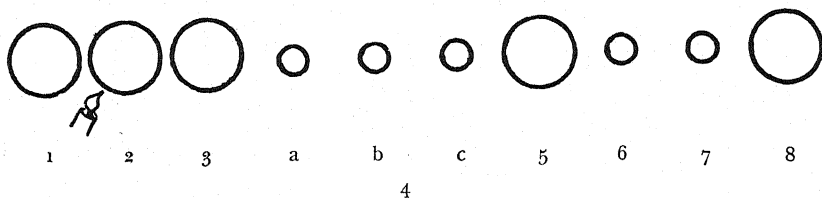


FIG. 14. Del Rio-Hortega's first variant of Achúcarro's method. 1. Water. 2. Tannic acid. 3. Water, ammonia. 4a, b, c. Silver oxide, ammonia. 5. Water. 6. Gold. 7. "Hypo." 8. Water.

*Note.* For more energetic staining the silver bath may be used without dilution. Alkalinity or precipitation weakens it.

- (6) *Wash.* Wash rapidly in two changes of distilled water.
- (7) *Reduce.* Plunge sections in 5 per cent neutral formalin.
- (8) *Tone.* Twenty minutes at room temperature.
- (9) *Fix.* In hyposulphite of soda.
- (10) *Wash.*
- (11) *Dehydrate, clear and mount.*

#### IV. Achúcarro's Tannin-silver Method<sup>26</sup>

This method, published in 1911, precedes those of Cajal and Del Rio-Hortega. It gave good results at times but was rather unreliable. Certain modifications were used in practice by Achúcarro particularly for protoplasmic astrocytes and these were published by his pupil Del Rio-Hortega<sup>27</sup> briefly as follows:

##### 1. The Method.

- (1) *Harden.* Pieces not over 2 to 3 mm. in thickness are placed in 20 per cent formol to which enough ammonia has been added to give an alkaline reaction with litmus paper.
- (2) *Section.* Frozen sections not over 10 $\mu$  in thickness.
- (3) *Mordant.* The sections, well flattened, are heated in 10 per cent solution of pure tannic acid for ten minutes, preventing bubbles.
- (4) *Wash.* After mordant has cooled wash the sections in water containing a few drops of ammonia until they become flexible.

<sup>26</sup> Achúcarro, N. *Bol. d. l. Soc. Esp. d. Biolog.*, 1: 139, 1911.

<sup>27</sup> Del Rio-Hortega, P. *Trab. d. Lab. d. Invest. Biol. d. Univ. Madrid*, 14: 181, 1916.

(5) *Impregnate*. Place sections in bath made by adding 3 to 4 drops of Bielschowsky's ammoniacal silver (solution k) to 10 c.c. distilled water. Remove the sections when the white matter begins to take on color.

(6) *Reduce*. Place in formalin prepared as for the hardening, for five minutes. This method demonstrates in general with great distinctness protoplasmic constituents such as granulations, centrosomes, myofibrils, glia fibrils and mitochondria, in addition to neuroglia and myelin sheaths. The *first* variant of Hortega described below, however, demonstrates the same structures more consistently.

## 2. Modification of Del Rio-Hortega. *The "First Variant" Method* (Fig. 14).

*Harden* at least ten days in 10 per cent formalin.

*Section*. Frozen sections.

(1) *Wash*. Water.

(2) *Mordant* the sections (cut on the freezing microtome) in 3 per cent tannic acid solution at 50°C. for five minutes.

(3) *Wash* them in 20 c.c. distilled water containing 4 drops of ammonia until their flexibility and elasticity return.

(4) *Impregnate* by passing the sections in groups of 3 or 4 through three dishes, A, B and C, arranged in series and each containing 10 c.c. distilled water and 1 c.c. ammoniacal silver (12 to 15 drops), (k). Leave the sections in first silver until the solution takes on a dark color, then pass them into the next. In the last solution the sections should be a deep yellow color while the solution itself is nearly colorless. A fourth dish of solution may be used if necessary.

(5, 6, 7, 8. Numbers refer to those used in Fig. 14.) *Wash*, tone in gold and fix in hyposulphite; wash. This is a good cytological stain for various purposes and may be used particularly for fibrous astrocytes.

The second and third variants of this method were devised by Del Rio-Hortega for staining collagenous fibers of connective tissue. However, the third variant also stains fibrous neuroglia but will not be detailed here. The fourth variant described by him at a later time is a useful method for protoplasmic astrocytes.

## 3. Modification of Del Rio-Hortega.<sup>28</sup> *The "Fourth Variant" Method*.

*Harden* in formalin for a long or short period. Cut sections as usual and place them for a few minutes at 45 to 50°C. in tannin-ammonium-bromide mordant (solution l). Wash in ammonia water until the sections are flexible and transparent. Impregnate in ammoniacal silver solution diluted as above until they are yellowish. Wash for a few seconds in water and reduce in 20 per cent formalin which has been neutralized by the addition of chalk some days before. Wash, tone and fix as above.

<sup>28</sup> In a personal communication Percival Bailey states that he has found this method very useful to demonstrate cytoplasmic outlines of cells in brain tumors.

## C. OLIGODENDROGLIA

V. Del Rio-Hortega's Silver Carbonate Method for  
Oligodendroglia<sup>29</sup> (Fig. 15)

*Harden.* Twelve to forty-eight hours in formalin-ammonium-bromide solution (c).

*Bromurate.* Heat block in fresh hardening solution (c) ten minutes at 45 to 50°C.

*Section.* Cut frozen sections at 15 to 20 $\mu$ .

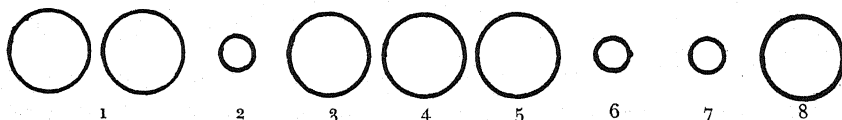


FIG. 15. Del Rio-Hortega's silver carbonate method for oligodendroglia. 1. Water. 2. Silver carbonate (strong). 3. Water. 4. 1 per cent formol. 5. Water. 6. Gold. 7. "Hypo." 8. Water.

(1) *Wash.* Wash in two changes of distilled water. Add 10 drops of ammonia to the first water.

(2) *Impregnate.* Place sections in strong silver carbonate solution (m-1) and leave one to five minutes as determined by trial.

(3) *Wash.* Agitate gently for about fifteen seconds.

(4) *Reduce.* Plunge sections into 1 per cent formalin. Do not agitate here.

(5-8) *Wash, tone* sections as usual until gray, fix in "hypo," wash, dehydrate, clear and mount.

*Result.* Oligodendroglia should be stained selectively. There may be faint staining of astrocytes and microglia may be well stained.

1. First Modification of Penfield.<sup>30</sup>

(1) *Harden.* In ammonium-bromide solution two to forty-eight hours. Blocks should not be more than 3 mm. in thickness. Place blocks in 95 per cent alcohol thirty-six to forty-eight hours.

(2) *Wash.* Wash blocks about four hours to get rid of alcohol in changes of distilled water in large volume. About four times as long in the water as is taken for the block to sink to the bottom is sufficient.

(3) *Section.* Cut on freezing microtome. If alcohol is not sufficiently washed out the cutting will be difficult.

(4) *Wash.* Pass through two changes of distilled water.

(5) *Impregnate.* Leave sections in strong silver carbonate fifteen minutes to two hours as determined by trial. The most favorable time to remove sections is when they are just beginning to turn brown.

<sup>29</sup> Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 1921.

<sup>30</sup> Penfield, W. *Brain*, 47: 430, 1924.

(6) *Reduce.* Plunge sections directly in 1 per cent formalin and agitate them at once. Wash, tone, fix, etc., as above.

*Result.* This method was successful particularly when using rabbit

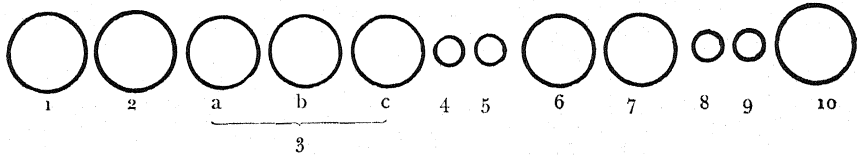


FIG. 16. Penfield's combined oligodendroglia and microglia method. 1. Ammonia. 2. Hydrobromic acid, 5 per cent. 3a, b, c. Water. 4. Sodium carbonate, 5 per cent. 5. Silver carbonate (weak). 6. Formol, 1 per cent. 7. Water. 8. Gold. 9. "Hypo." 10. Water.

material. The alcohol lends a clear smooth appearance to the sections. Microglia cells are often stained as well as oligodendroglia. The method, like the method of Hortega, is variable, although the results are brilliant when successful. It is suitable chiefly for experimental material.

We have found that oligodendroglia can be stained in every case without fail if the above modification be employed omitting the immersion of blocks in alcohol and proceeding as follows: Inject the formalin-ammonium-bromide into the internal carotid. Remove a block from the hardening solution at the end of two hours and run sections through. If not successful repeat the following morning, and if necessary repeat again after twelve to twenty-four hours, removing each time a fresh block from the fixative. The duration of fixation seems to be of the utmost importance but is difficult to standardize.

The following modification we would recommend for routine use, as it gives uniform results and may be applied to formalin or formalin-ammonium-bromide fixed material even after a considerable period of time:

**2. Second Modification of Penfield (Fig. 16). *The Combined Oligodendroglia and Microglia Method.*<sup>31</sup>**

*Harden.* Tissue in formalin or formalin-ammonium-bromide solution for an indefinite period. About a week in formalin gives excellent results.

*Section.* Cut sections at  $20\mu$  on the freezing microtome and receive them in 1 per cent formalin or distilled water.

(1) *Deformalinize.* Place sections in dish of distilled water to which 10 to 15 drops of strong ammonia have been added and cover so as to prevent escape of ammonia. Leave here overnight to remove formalin.

(2) *Bromurate.* Transfer sections directly to Globus' hydrobromic acid in 5 per cent solution (5 c.c. of 40 per cent hydrobromic acid plus 95 c.c. distilled water). Place in incubator at  $38^{\circ}\text{C}$ . for one hour.

<sup>31</sup> Penfield, W. *Am. J. Path.*, 4: 153, 1928.



- (3) *Wash.* Pass through three changes of water (a, b, c).
- (4) *Mordant.* Place sections in 5 per cent solution of sodium carbonate for one hour. (Sections may remain here five to six hours without ill effect.)
- (5) *Impregnate.* Pass sections with or without washing direct to Hortega's silver carbonate, weak solution (m-2) and leave them here three to five minutes or until they turn a smooth gray when transferred to the reducer. Control by taking out a section at intervals of one to two minutes and examining under the microscope. At times good results are obtained by leaving the sections in the silver solutions until they turn a light brown.
- (6) *Reduce.* Place in 1 per cent formalin and agitate.
- (7) *Wash.* Distilled water.
- (8) *Tone.* Leave in gold chloride (1 to 500) at room temperature until sections are a smooth bluish gray.
- (9) *Fix.* Hyposulphite of soda (5 per cent) as usual.
- (10) *Wash,* dehydrate, clear and mount.

By this method both microglia and oligodendroglia may be stained with a considerable degree of consistency. The morphological differences of the two types of cells make it quite easy to distinguish them. This differentiation is even easier when either type of cell has undergone some pathological change. There may be at times faint staining of astrocytes particularly if the sections are left too long in silver.

#### D. MICROGLIA

(Mesoglia, Hortega cells, Hortega's third element.)

#### VI. Del Rio-Hortega's Silver Carbonate Method for Microglia<sup>32</sup>

(p. 494)

*The Method*<sup>33</sup> (Fig. 17).

*Harden.* Blocks about 3 mm. in thickness should be hardened in formalin-ammonium-bromide solution (c) for two to three days at room temperature.

*Bromurate.* Place blocks in fresh formalin-ammonium-bromide in a small covered glass dish and heat to about 50°C. for ten minutes.

*Section.* Cut sections at once in the freezing microtome at 20 to 25 $\mu$ , receiving the sections in distilled water.

(1) *Wash.* Pass sections through three changes of distilled water (a, b, c, Fig. 19) adding to the second change 4 or 5 drops of ammonia to remove the formalin.

<sup>32</sup> Del Rio-Hortega, P., and Asua, F. J. de. *Arch. d. cardiol. y hematol.*, 2: 161, 1921.

<sup>33</sup> The method is first given as outlined in the cited articles, and with certain minor additions which were in use in the Laboratorio de Histopatologia de la Junta para Ampliacion de Estudios in the year 1924 when it was the privilege of one of us (W. P.) to work with the author of this method. It is a pleasure to acknowledge our indebtedness to this master of histological technique.

(2) *Impregnate.* Place four or five sections in Hortege's silver carbonate "weak solution" (m-2).

(3) *Reduce.* Remove one from the silver at the end of twenty seconds and

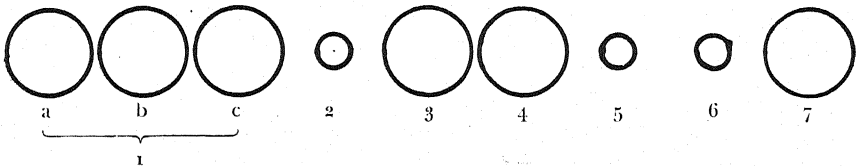


FIG. 17. Del Rio-Hortega's silver carbonate method for microglia. 1a. Water. 1b. Water, ammonia. 1c. Water. 2. Silver carbonate (weak). 3. Formol, 1 per cent. 4. Water. 5. Gold. 6. "Hypo." 7. Water.

plunge directly into 1 per cent formalin. Agitate the section at once by blowing briskly on the surface of the liquid. Remove a second at forty-five seconds and another at two minutes. The sections should promptly change to smooth gray color. One minute is long enough in the reducer. If sections are brownish the duration in silver has probably been too long and neuroglia astrocytes will be found stained as well as microglia.

It is well to float sections on a slip and examine under the microscope. The microglia cells are seen as small black spider shapes. If neuroglia astrocytes are stained they are likely to be tan and, of course, larger. When the optimum duration of silver impregnation has been determined, ten to twenty sections at a time may be carried through it and into formalin with a similar result.

(4) *Wash.* Distilled water.

(5) *Tone.* Leave the sections in gold chloride solution (h) until they become an even gray with no yellow shade. If left until they are slightly purple the stain is intensified.

(6) *Fix.* Place in hyposulphite of soda (5 per cent) about a minute until they are flexible.

(7) *Wash.* Place sections in water. They may be preserved here or better in 1 per cent formalin for long periods. They may be counterstained if desired before continuing to dehydrate; clear and mount in Canada balsam. For the general background and connective tissue picro-fuchsin solution as for Van Gieson's method may be used, or picro-indigo stain of Cajal.

If the procedure is not successful place the sections in absolute alcohol for about a half hour, then wash, place in silver solution and proceed as usual. Anhydrous sodium sulphite, a pinch in 50 c.c. of water, may be used in a similar empirical fashion with occasional resultant improvement. Finally, the preliminary use of strong ammonia or strong pyridine or a mixture of the two with water in equal parts may influence the outcome favorably.

Better results are occasionally obtained by heating the silver bath to 50°C. and leaving sections here until they are of an amber color. If the solution becomes turbid before this color has been reached they have

been improperly washed. Sections are next washed rapidly in distilled water before reduction. This variation is particularly helpful when working with the cerebrum of dogs according to Gallego. We have

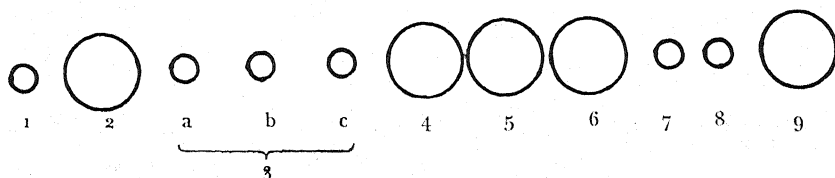


FIG. 18. Del Rio-Hortega's 1927 method for microglia. 1. Pyridine, ammonia, water. 2. Water. 3a, b, c. Undiluted silver carbonate. 4. Water. 5. 1 per cent formol. 6. Water. 7. Gold. 8. "Hypo." 9. Water.

found this alternative also occasionally successful in old formalinized material.

*Failure* may be due to imperfectly distilled water, to over-formalinization or unsuitable formalin and to impurity in the sodium carbonate from which the silver carbonate is made. If the material is from the rabbit's brain a successful result is almost invariable. With mouse, cat, dog and human material the method gives variable results. Morphologically microglia seems to be exactly the same in all these species.

1. **Del Rio-Hortega's 1927 Method** (Fig. 18). More recently Del Rio-Hortega has given rules for the application of the method to macrophages as seen throughout the body in various tissues as well as to microglia.<sup>34</sup> The most generally applicable variant is as follows:

*Harden.* Blocks in formalin-ammonium-bromide for two to eight days. (Results may also be good if fresh material is fixed for a short time in formalin or for a long period in formalin-ammonium-bromide. For macrophages in general 10 per cent formalin is preferable.)

*Section.* Frozen sections at  $20\mu$ , or imbed in gelatin if preferred.

(1) *Preparation.* Place sections ten minutes or more in a mixture of equal parts of pyridine, ammonia and distilled water (5 per cent crystallized sodium sulphite may be substituted for this mixture).

(2) *Wash.* This may be omitted.

(3) *Impregnation.* Undiluted silver carbonate (m-3) is placed in three small glass dishes and the sections are passed through them in series, remaining thirty seconds in the first, one minute in the second and one minute or more in the third, depending on the temperature. Trial sections may be run through as in the original technique.

(4) *Wash.* Wash rapidly (ten to fifteen seconds) in distilled water. At times this is unnecessary. Too much washing renders the staining granular.

(5) *Reduce.* Pass to 1 per cent formalin, agitating the sections gently. Sometimes moving the sections in the reducer in this way may be found detrimental,

<sup>34</sup> Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 27: 199, 1927.

as the silver is washed out from the macrophages. The same thing may happen with the preliminary washing.

(6, 7, 8, 9) *Wash, tone, wash and fix* as above.

With this method Hortega has secured good results in staining microglia and also epithelioid cells, cells of Langerhans, Kupffer cells of the liver and in general the cells included with the reticulo-endothelial system of the kidney, the tonsil, the lymphatics, lungs, etc.

Particularly good results with cat material were obtained by Hortega when he left the sections ten to fifteen minutes in undiluted silver carbonate solution and then placed the sections one by one in water where they must lie extended but without movement for about five minutes until the sections take on a pale yellow color. Reduce in 5 per cent formalin and complete as usual.

**2. Modification of Cajal. Silver Oxide Method.** Cajal has proposed a modification of this method. He substituted Bielschowsky's silver oxide solution (solution j) for the silver carbonate solution. He reports success with old formalin material, particularly pathological human material. In an earlier publication<sup>35</sup> Cajal described a similar method which also resembles that of his pupil Del Rio-Hortega under the name of "A Modification of the Method of Bielschowsky." We have used Bielschowsky's silver as recommended by Cajal but have always found it less satisfactory than Hortega's silver carbonate. The more recent description of Cajal's modification is as follows:

(1) *Harden.* Blocks as usual in formalin-ammonium-bromide solution, human material for thirty to forty-five days, animal material less than four days.

(2) *Mordant.* The sections are placed in reinforcer (i) in incubator a few hours.

(3) *Wash* rapidly three times.

(4) *Impregnate.* Place in silver oxide solution (j) diluted with an equal amount of water to which pyridine has been added. Leave here until they take on a pale straw color or heat until they become the color of "weathered straw."

(5) *Reduce.* In 5 per cent formalin and complete as usual.

## E. SPECIAL METHODS

**1. Modification of Del Rio-Hortega for Perivascular Glia of Andriezen.**<sup>36</sup> The perivascular glia of Andriezen are astrocytes of both the fibrous and the protoplasmic types which are closely applied by their body cytoplasm to vascular adventitia while the expansions radiate off into the surrounding tissue at a distance. These cells, which were

<sup>35</sup> Cajal, S. Ramón y. *Trav. d. Lab. d. Rech. Biol. d. l'Univ. d. Madrid*, 23: 157, 1925.

<sup>36</sup> Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 25: 184, 1925.

well described by Andriezen, curiously enough seem to have staining affinities similar to those of oligodendroglia and microglia.

Del Rio-Hortega has pointed out that the perivascular glia is well stained by the following technique:

The tissue should be fresh and preferably hardened not more than a day or two. Wash frozen sections from formalin-ammonium-bromide fixed material ten to twenty minutes in pure pyridine. Wash in two changes of distilled water without eliminating all the pyridine.

The sections may then be stained according to the first method of Hortega for microglia (p. 509) or the alcohol modification of Penfield for oligodendroglia (p. 510).

**2. Modification of Del Rio-Hortega for Neuroglia Pigment.** This method also demonstrates the pigment if present in nerve-cells and in microglia. It shows the pigment present both under normal conditions and as the result of pathological processes.

(1) *Harden.* In formalin or formalin-ammonium-bromide for an indefinite period.

(2) *Bromurate.* Heat the blocks in formalin-ammonium-bromide ten minutes at 45 to 50°C.

(3) *Section.* On freezing microtome.

(4) *Wash.* In strongly ammoniacal water.

(5) *Prepare.* Sections in sodium sulphite, 5 per cent solution for some minutes.

(6) *Impregnate.* Plunge sections directly into weak silver carbonate solution (m-2) and heat to 50°C. until sections are the color of tobacco.

(7) *Wash.* In water for one-half to one minute.

(8) *Wash.* In 95 per cent alcohol one-half minute.

(9) *Reduce.* In 1 per cent formalin.

(10) *Tone.* In gold chloride and reinforce by heating.

(11) *Fix.* In "hypo" 5 per cent.

*Wash and mount as usual.*

**3. Del Rio-Hortega's Methods for Gliosomes and Mitochondria.** Gliosomes are small rounded granules found in neuroglia (both astrocytes and oligodendroglia). They can be distinguished at times according to Hortega<sup>37</sup> from mitochondria; at other times it is impossible to make any such distinction. The whole question of the nature of the specific granules found in these cells must be left an open one for the present. It has been thought (Nageotte, Mawas, et al.) that these granules correspond to secretion granules and that neuroglia as a whole is a huge gland of internal secretion situated in a diffuse fashion through-

<sup>37</sup> Del Rio-Hortega, P. *Bol. d. l. Real Soc. Esp. d. Hist. Nat.*, 25: 34, 1925.

out the central nervous system. The method may be carried out in one of three ways.

(a) *The Method. Staining in the Cold (Mitochondria).*<sup>38</sup>

(1) *Harden.* Place blocks of 2 to 3 mm. thickness in formalin-iron-alum solution (n).

(2) *Section.* Cut sections on freezing microtome at about  $10\mu$ , taking care not to freeze the tissue too hard.

(3) *Wash.* Pass through two good-sized dishes of water. Add 5 to 6 drops of ammonia to the first water to render the sections more flexible.

(4) *Impregnate.* Silver carbonate solution (m-2) for about five minutes, depending on the temperature of the room.

(5) *Wash.* Distilled water for fifteen to thirty seconds. Do not move sections too much here. This is an important step. The water acts as a differentiator removing silver from the general tissue and leaving it only in the mitochondria. If this is not done carefully only the more voluminous and more energetically impregnated gliosomes are likely to be stained.

(6) *Reduce.* Formalin (1 to 200). For the study of mitochondria the section should turn a grayish color. Gliosomes are best seen in sections which take on a reddish hue. Wash.

(7) *Tone.* Leave sections in 1 to 500 gold chloride solution for about fifteen minutes or heat gently until the color is a little more intense.

(8) *Fix.* In "hypo" as usual.

*Result.* At least in dog and cat material the mitochondria of neuroglia are well stained as are sometimes similar granules in nerve-cells.

(b) *Staining in the Heat (Gliosomes).*

(1) *Harden.* Two to eight days in the formalin-iron-alum solution. (Tissue fixed for months in formalin-ammonium-bromide may be used if the sections are left twenty-four hours in 5 per cent solution of iron alum.)

(2) *Wash.* Two or three large dishes of distilled water to which ammonia is added followed by a wash in water without ammonia.

(3) *Impregnation.* Heat in silver carbonate (m-2) to which 2 or 3 drops of pyridine have been added at 45 to 50°C. shaking occasionally, until sections take on a light tobacco color.

(4) *Wash.* One to three minutes in distilled water.

(5) *Reduce.* Formalin (10 per cent).

(6) *Tone.* Fifteen minutes in the cold and then intensify the color by heating gently.

(7) *Fix.* As usual.

*Result.* This procedure is used chiefly to stain gliosomes. The ad-

<sup>38</sup> For procedure (a) and (c) the formalin uranium (solution o) may be used. This fixative and mordant gives particularly good results for protoplasmic astrocytes and even better for oligodendroglia.

vantage of this modification is that neuroglia bodies and expansions are stained without blotting out the granules.

It is stated that a reducer of 5 to 10 per cent formalin demonstrates gliosomes by preference; 1 per cent reducer tends to demonstrate the mitochondria of glia and in part those of nerve-cells; dilution of reducer to 1 to 400 produces splendid staining of mitochondria in neuron bodies and larger axons. For better staining of gliosomes in oligodendroglia dilute the silver solution and add about 20 drops of 95 per cent alcohol as well as pyridine to it.

(c) *Double Impregnation.*

(1) *Harden.* In formalin-iron-alum solution or in this solution plus 2 per cent of ammonium-bromide, three to four days.

(2) *Section and wash* as in (b).

(3) *Prepare.* Place sections in 2 per cent silver nitrate and heat gently.

(4) *Wash.* Rapidly.

(5) *Impregnate.* Weak silver carbonate one minute.

(6) *Wash.* Very rapidly.

(7) *Reduce.* Formalin 1 per cent.

(8) *Tone and fix* as in (b).

*Result.* This procedure succeeds at times when (a) fails and is particularly helpful in staining the cerebellar neuroglial cells of Bergmann, if formalin-uranium is used to harden the tissue. The procedure also demonstrates very well the granules of ependymal cells.

**4. Counterstaining of Microglia.** For staining of lipoid material G. Herxheimer's stain may be used. This is a saturated solution of scarlet red in 70 per cent alcohol and pure acetone. Staining takes place very quickly.<sup>39</sup>

For staining iron in macrophages Del Rio-Hortega follows the procedure outlined by Perls as follows:

*Counterstain of Microglia for Iron (Perls).*

(1) After staining the sections with silver and toning, gently heat them to 45 or 50°C. in a solution of 5 per cent potassium ferrocyanide for fifteen minutes.

(2) Place them in hydrochloric acid (10 per cent) at a temperature of about 25 to 30°C. for fifteen minutes.

(3) Wash well in two changes.

(4) Stain with Ziehl's fuchsin fifteen seconds.

(5) Wash well in water.

(6) Place in saturated solution of picric acid and leave there some seconds until sections darken.

(7) Differentiate in 95 per cent alcohol until sections become pale red.

(8) Clear in carbol-xytol-creosote (f).

(9) Mount in balsam.

<sup>39</sup> Mallory, F. B., and Wright, J. H. *Pathological Technique*, Phila., 1924.

When microglia contains much iron, it will be demonstrated well out in the expansions. In their ameboid forms a large amount of iron will be found in these cells.

## F. SOLUTIONS

*a. Distilled Water.* It is our custom to use doubly distilled water for all solutions and washings, the second distillation being done in glass. In the laboratories of Madrid the taps supply water straight from the melting snows of the Guadarramas. This is not distilled for routine use.

*b. Ten Per Cent Formalin.*

Merck's blue label 40 per cent formaldehyde.....	10 c.c.
Distilled water .....	90 c.c.

We have not found it necessary to add chalk to this formalin<sup>40</sup> as it is very nearly neutral. When using other makes of formol we have added chalk to the stock. We believe that Cajal used formalin prepared by Merck or Kahlbaum to which he added chalk. It is the custom of Del Rio-Hortega to use Merck's formalin. Highly acid formalin is prejudicial to many of the reactions.

*c. Cajal's Formalin-ammonium-bromide Solution (F.A.B.).*

Formalin (Merck's blue label, 40 per cent).....	14 c.c.
Ammonium bromide .....	2 gm.
Distilled water .....	86 c.c.

*d. Cajal's Gold Chloride and Sublimate Solution.* Prepare fresh bath each time with scrupulously clean glassware as follows:

Mercury bichloride crystals .....	0.5 gm.
Gold chloride (Brown, Merck) 1 per cent.....	10 c.c.
Distilled water .....	50 c.c.

Pulverize the sublimate and add it to the 50 c.c. of water and place over alcohol flame so as to dissolve rapidly but do not overheat, or place in hot water bath. When dissolved add gold chloride to solution which is still hot. Filter the mixture. Cajal has warned against buying the powdered preparation of sublimate. The gold chloride keeps well in a brown bottle for long periods. Cajal advised in his last formula a smaller concentration of gold than here given, i. e., 6 c.c.

*e. Cajal Fixing Bath.*

Sodium hyposulphite .....	5 gm.
Water .....	70 c.c.
Alcohol (95 per cent).....	30 c.c.
Concentrated solution of sodium bisulphite.....	5 c.c.

<sup>40</sup> The pH of the Merck's blue label 40 per cent formalin has been about 6.8. The 10 per cent solution made from this has had an average pH of 6.2.



f. *Carbol-xylol-creosote Mixture.*

Creosote .....	10 c.c.
Phenol .....	10 c.c.
Xylol .....	80 c.c.

g. *Del Rio-Hortega's Silver Carbonate Solution (Lithium) for Astrocytes.*

Silver nitrate (10 per cent).....	5 c.c.
Lithium carbonate (saturated solution).....	20 c.c.
Ammonium hydroxide q.s. to dissolve precipitate	
Distilled water ad .....	75 c.c.
Filter and keep in a brown bottle	

When the first two chemicals are combined silver carbonate comes down as a voluminous precipitate. The ammonia should then be added drop by drop stirring all the while until the precipitate disappears and ammoniacal silver goes into solution. Care must be taken to add no more ammonia than just enough to cause this resolution. A small amount of black dustlike precipitate will remain undissolved and may be filtered off. The solution may usually be kept for long periods.

This is the preparation employed by Del Rio-Hortega more recently. In the original publication of the method (p. 505) Del Rio-Hortega recommended silver nitrate (10 per cent) to which was added an equal or larger amount of saturated solution of lithium carbonate. He then decanted the liquid and washed the precipitate with 50 c.c. of distilled water. He decanted again and then added 15 to 20 c.c. water and followed with ammonia sufficient to dissolve the precipitate, after which water was added so as to increase the total volume to 50 c.c.

h. *Gold Chloride Solution for Toning.*

Gold chloride (yellow) .....	1 gm.
Distilled water .....	500 c.c.

It is not necessary to use the more expensive brown gold for toning; in fact the yellow variety seems to serve the purpose better.

i. *Cajal's Reinforcer.*

Neutral formalin .....	30 c.c.
Ammonium bromide .....	3 gm.
Distilled water .....	70 c.c.

j. *Bielschowsky's Ammoniacal Silver Bath as Prepared by Cajal.*

Silver nitrate, 1 per cent.....	20 c.c.
Sodium hydroxide, 40 per cent.....	22 drops

Wash the precipitate with distilled water six times.

Add to the precipitate at a temperature of 22°C.

Distilled water .....	110 c.c.
Ammonium hydroxide .....	4 c.c.

Stir until precipitate is dissolved. Keep in dark bottles. The test for the activity of this silver oxide solution is the rapidity with which sections turn dark brown when placed in it at room temperature.

*k. Bielschowsky's Ammoniacal Silver Bath as Prepared by Del Rio-Hortega for Achúcarro's Method.*

Silver nitrate 10 per cent. ....	30 c.c.
Sodium hydroxide .....	40 drops

Wash the precipitate ten or twelve times in distilled water, using a liter at least. Add to the precipitate 50 c.c. of distilled water. Add to this ammonia in just sufficient amount to dissolve the precipitate, stirring without too great violence. Add no more ammonia after the solution smells of it. Add distilled water so as to bring total up to 150 c.c.

*l. Mordant for Hortega's Fourth Variant.*

Tannin .....	3 gm.
Ammonium bromide .....	1 gm.
Distilled water .....	100 c.c.

*m. Del Rio-Hortega's Ammoniacal Silver Carbonate Solution.*

1. Strong solution.

Solution of silver nitrate (Merck) 10 per cent. ....	5 c.c.
Solution of sodium carbonate (pure) 5 per cent. ....	20 c.c.
Ammonium hydroxide (sufficient to dissolve precipitate)	
Distilled water up to .....	45 c.c.

The ammonium hydroxide, as indicated above, should be added drop by drop until the precipitate is just dissolved, stirring the solution all the while. Finally, filter and place in a dark bottle, where it will keep for long periods.

2. Weak solution. Is prepared in the same way, except that distilled water is added up to 75 c.c.

3. Undiluted silver carbonate is made up the same way but no water is added after the precipitate is dissolved with ammonia.

*n. Del Rio-Hortega's Formalin-iron-alum Solution.*

Formalin (40 per cent) .....	10 c.c.
Distilled water .....	90 c.c.
Iron alum (pure) .....	6 to 8 gm.
Filter	

*o. Del Rio-Hortega's Formalin-uranium Solution.*

Formalin (40 per cent) .....	10 c.c.
Uranium nitrate .....	1 to 2 gm.
Distilled water .....	90 c.c.

## CHAPTER VIII

### PROTOZOOLOGICAL METHODS

D. H. WENRICH

Introduction 522. Examination of living protozoa 522. Temporary killing and staining methods 529. Permanent mounts 530.

#### I. Introduction

In order to gain a full knowledge of any particular kind of Protozoa it is necessary to study them in the living condition in as normal an environment as possible, and, in addition, to employ a wide range of technical aids. In the present section suggestions are offered for (1) the collection and examination of living Protozoa, (2) intravital staining, (3) temporary methods of killing and staining and (4) preparation of permanent mounts. As a section of a general work, the presentation is not intended to be exhaustive. Those methods which seem to the author to be of the greatest usefulness have been selected. Some familiarity with laboratory methods is presupposed.

#### II. Examination of Living Protozoa

1. **Free-living Protozoa.** *a. Collection.* Collection of free-living Protozoa may be done by simply dipping up a quantity of water from a stream, pool or larger body of water, or by using some form of special apparatus.<sup>1</sup> Where variety of species is desired, it should be remembered that Protozoa are to be found in moist soil, on and in the bottom deposits of water bodies and attached to all kinds of submerged objects, including growing water plants as well as swimming freely in the water, and collections from all these sources should be made. If the sample collected is to be representative of a body of water, such as a reservoir, then it should be taken out away from shore and away from any special environmental condition such as floating objects.

*b. Concentration Methods.* These are frequently employed, usually involving some kind of filtering device. The simplest method is the following: Pass the sample of water through filter paper in an ordinary funnel. When 10 to 20 c.c. of water remain in the funnel quickly pour

<sup>1</sup> Whipple, G. C. *The Microscopy of Drinking Water*. Ed. 4. N. Y., 1927.  
Ward, H. B., and G. C. Whipple. *Freshwater Biology*. N. Y., 1918.

the residue into a vessel and examine as a concentrate. By measuring the original sample and counting all the individual specimens of each kind in the concentrate, this method can be made quantitative. Ordinary filter paper will permit many of the smaller forms to pass through. A hard filter paper may be used, but filtering is slowed down and the funnel will need to be agitated frequently to prevent the Protozoa from becoming attached to the paper. A suction exhaust will hasten filtering but may injure the more delicate species.

*Concentration in the Field.* Select a wide-mouthed bottle or jar provided with a stopper having two holes through it. In one hole insert the stem of a small funnel in an inverted position with its broad end covered with bolting cloth. In the other hole in the stopper insert a larger funnel in an upright position. Put the stopper in place and pour the water into the upright funnel. When the bottle is full the water will filter out through the smaller funnel, the organisms being retained by the bolting cloth. A relatively large volume of water may thus be concentrated for transportation to the laboratory.<sup>2</sup>

Towing with a tow net is essentially a method of concentration. By using certain forms of tow net and observing certain rules, this method may be made quantitative.<sup>3</sup>

*The Sedgwick-Rafter method* is designed to give quantitative results. Special apparatus consists, first, of a graduated funnel of 500 c.c. capacity which is cylindrical through most of its length. Near the lower end it tapers to a much narrower cylinder which is also graduated. The lower end is provided with a perforated rubber stopper into the opening of which is inserted a glass tube up to the upper surface of the stopper. At the lower end of the glass tube a rubber tube provided with a clamp is attached. The upper end of the stopper is covered with a disk of bolting cloth and on this enough fine sand is poured to make a layer of  $\frac{3}{4}$  to  $1\frac{1}{2}$  inch in depth. The sand should be fine enough to pass through a sieve having 60 meshes to the inch. Two other special pieces of apparatus are (1) the counting cell, which is a microscopical slide with a brass rim cemented to it to make a cell exactly 1 mm. deep, and (2) the ocular disk, which has a large square on it ruled into 100 smaller squares. This disk is placed in the ocular of the microscope and such a combination of lenses and tube length is chosen that one side of the large square corresponds to 1 mm. on the stage of the microscope. (Calibrate with a stage micrometer.) When the counting cell is in place, by counting all the organisms within the large square of the ocular disk, one actually

<sup>2</sup> Hall, W. E. *J. Roy. Micr. Soc.*, p. 46, 1924.

<sup>3</sup> See Ward and Whipple. *Loc. cit.* p. 389.

counts the number in a cubic millimeter, since the cell is just 1 mm. deep. The apparatus is used in the following manner:

Having secured a carefully collected sample of water, stir it to obtain an even distribution of the organisms, pour a little water into the funnel to wet the sand and fill the funnel up to the 500 c.c. mark. Next remove the clamp from the outlet tube and allow filtering to proceed. When a small quantity of water, say 1 c.c. remains in the lower end of the funnel, replace the clamp to stop the filtering. Place a tumbler or beaker under the funnel and remove the stopper, allowing the sand and concentrate to fall into the tumbler. Quickly wash the inside of the funnel with a small measured quantity (say 9 c.c.) of sterile water to remove attached organisms. Next gently stir the concentrate and sand to free the organisms from the sand, and after letting it stand for a few seconds so that the sand may settle to the bottom, decant the water containing the organisms into another container. Again stirring the concentrate to secure even distribution of the organisms, take some of it up with a pipette and place it in the counting cell.

To prevent overflowing place the cover glass over most of the cell before the concentrate is added. When the cell is full adjust the cover glass and place the cell on the stage of the microscope. Using a mechanical stage to prevent duplication of fields, count all the organisms of each kind in a definite number of ocular squares, each in a different field of the microscope, and record the numbers. The number of individuals in each square is really the number in cubic millimeters of the concentrate. The results are expressed in terms of the number of each kind of organism per cubic centimeter of the original sample. In order to bring the actual counts into this form the following formula is used:

$$N = \frac{t}{n} \times \frac{1000 c}{v}$$

in which  $N$  is the number per cubic centimeter of original sample,  $t$  is the total number of a particular kind counted in all the squares,  $n$  is the number of squares counted,  $c$  is the number of cubic centimeters of the concentrate after washing the funnel and  $v$  is the number of cubic centimeters of the original sample. If we suppose that 20 squares were counted and that 10 c.c. was the amount of concentrate after filtering 500 c.c. of the original sample, and if we substitute these quantities in the above equation, we get

$$N = \frac{t}{20} \times \frac{1000 \times 10}{500}$$

which cancels out to  $N = t$ , making calculation very simple.

This calculation needs to be made for each kind of organism recorded and, in addition, a survey should be made of the entire counting cell to find any kinds that may have been missed in the series of squares counted. Such a random sampling method naturally has inherent defects, but in spite of these, tests have shown that parallel determinations from

the same sample do not usually differ more than 10 per cent from each other.<sup>4</sup>

*c. Examination.* If the collected sample of water is allowed to stand undisturbed in a vessel for an hour or more the Protozoa tend to distribute themselves in different parts of the vessel depending on their several tropic responses. Those that are positively phototropic will be found on the most brightly illuminated side. Geotropism will send some to the top and others to the bottom. If a random sampling method is used to obtain quantitative results, then the sample or concentrate should be thoroughly stirred before examination.

In ordinary work, proceed as follows: place a drop of water containing the organisms on a slip and cover with a cover glass. A depression slide may be used but is not necessary. If there are no objects in the drop capable of supporting the cover glass, add pieces of fine glass rods or tubing, or pieces of cover glass to the drop, or attach small lumps of wax to the corners of a square cover glass to prevent crushing the animals. If the cover glass is unsupported, evaporation will deplete the film of water and the organisms will be distorted by pressure. This, however, is a decided advantage for observing certain details, such as the collecting canals of the contractile vacuoles of *Paramecium*. At one time it was a common practice to compress organisms in a special compressor for examination of their finer structures.

Compensate for evaporation by adding water at the edge of the cover glass with a dropper, or seal a short shell vial to one end of a slip with paraffin or cement to act as a water reservoir, and arrange a small string or coarse thread to act as a capillary siphon to carry water from the reservoir over to the fluid under the cover glass.

*Examination in cultures* is possible where Protozoa have been cultured in a Syracuse watch glass or similar small dish. Place the dish on the stage of the microscope and examine the animals directly. Low power objectives will not need to touch the water, but for higher magnification, water-immersion objectives are available. This method is especially advantageous for sedentary forms and for tracing development of a culture.

*A hanging drop* is convenient for confining one or a small number of Protozoa in a limited amount of fluid for prolonged observation. Place a small drop of the culture containing the organisms in the middle of a round cover glass, or isolate a single animal in such a drop and invert the cover over the concavity of a depression slide and seal with vaseline, paraffin, or other cementing substance, or seal a glass ring to an ordinary slip to make a chamber over which the cover with the drop is inverted

<sup>4</sup> Whipple, G. C. *Loc. cit.* p. 389.

and sealed. Special forms of culture slides are also to be had from the supply houses.

*Slowing the movements* or restricting the activities of ciliates and flagellates: Either (1) isolate in a small drop of fluid, e. g., a hanging drop, or (2) add a little cotton wool to the drop on the slide, or (3) thicken the medium by adding 2 per cent or 3 per cent gelatin, quince-seed jelly, cherry-tree gum, or 25 per cent gum acacia, or (4) add some anesthetizing agent.

*Anesthetization.* (1) Spray alcohol, ether or chloroform on the water, or (2) drop small crystals of menthol on the water, or (3) add one of the following aqueous solutions to the organisms on a slide or in a dish: 0.1 per cent nicotine, 0.1 per cent to 0.2 per cent chloral hydrate, 1.0 per cent cocaine hydrochlorate, 1.0 per cent cocaine hydrochloride, 1.0 per cent magnesium sulphate, or magnesium chloride, or 0.1 per cent chloretone. Cole and Richmond<sup>5</sup> state that 1 drop of 0.12 per cent chloretone added to a drop of equal size of culture containing paramecia, giving a concentration of about 0.056 per cent chloretone, will anesthetize the paramecia in about ten minutes and will keep them quiet for two days or longer up to ten days.

**2. Associated Protozoa.** *a. Ectozoic Protozoa.* Examine if possible while these are still attached to their hosts. When this is impossible examine them in their native medium, which is, of course, that of their hosts.

*b. Endozoic Protozoa.* Examine in the body fluids of the hosts or in some fluid which is isosmotic, or nearly so. Examine blood-inhabiting species in the blood itself by placing a drop of blood on the slide and covering with a cover glass, or dilute the blood with some saline diluent such as physiological salt solution, Ringer's solution (to 100 c.c. of distilled water add 0.8 gm. NaCl, 0.02 gm. CaCl<sub>2</sub>, 0.02 gm. KCl, 0.02 gm. NaHCO<sub>3</sub> and (optional) 0.1 gm. dextrose); Locke's solution (to 100 c.c. of distilled water add 0.9 gm. NaCl, 0.04 gm. KCl, 0.02 gm. CaCl<sub>2</sub>, 0.001 to 0.003 gm. NaHCO<sub>3</sub> and 0.1 gm. dextrose), or a solution made by adding 0.5 gm. of sodium citrate and 0.3 gm. sodium chloride to 100 c.c. of distilled water. Examine tissue inhabiting species by teasing out the tissues in one of the above saline solutions.

*Intestinal Protozoa.* Feces or intestinal contents usually require dilution before examination. In most cases, a sodium chloride solution of 0.4 per cent to 0.6 per cent or Drbohlav's<sup>6</sup> modified Ringer's solution (to 100 c.c. of distilled water add 0.6 gm. NaCl, 0.01 gm. KCl, 0.01 gm. CaCl<sub>2</sub>, and 0.01 gm. NaHCO<sub>3</sub>) is preferable to stronger solutions, and

<sup>5</sup> Cole, W. H., and Richmond, E. *Proc. Soc. Exp. Biol. & Med.*, 22: 231, 1925.

<sup>6</sup> Drbohlav, J. J. *Ann. de Parasitol., Hum. et Comp.*, 3: 349, 1925.

Cleveland<sup>7</sup> states that 0.2 per cent sodium chloride is better for the Protozoa in certain tropical termites.

Kirby<sup>8</sup> recommended 67 per cent Locke's solution as an observation medium for other flagellates of termites. For prolonged observation of intestinal Protozoa, the cover glass should be ringed with vaseline, soft paraffin or paraffin oil to prevent evaporation. The amount of dilution should be adjusted to allow plenty of light to pass through for microscopic observation. The exact amount can best be determined by experience. It may be noted that when intestinal and other endozoic Protozoa are examined in a saline medium without ringing the cover glass, they will frequently become immobilized when they approach the edge of the cover. Such gelated specimens of ciliates and flagellates often reveal the number and character of their locomotor organs which can be made out only with difficulty in the motile animal. Examination with the dark field will aid in determining the number of flagella of flagellates.

*Warm-blooded Host-inhabiting Protozoa* often quickly lose their motility or degenerate when the temperature is reduced below that normal for the hosts. For maintaining a constant temperature, use a warm stage or place the microscope in a warm box. There are several types of warm water and electric warming stages on the market.

**3. Intravital Staining.** Intravital staining methods are equally applicable to free-living and associated Protozoa. For free-living forms, the dyes are usually dissolved in distilled water or in the native medium of the organisms, while for endozoic species they are dissolved in physiological salt solution. The agents used are either finely divided particles in suspension or basic dyes.

*Methods for Food Vacuoles in Ciliates.* Demonstrate by placing non-toxic colored particles, such as finely divided carmine or finely divided carbon ("Chinese ink" or "India ink") into the culture medium. Such colored particles are often ingested in the same way that food particles are and the food vacuoles thus become readily visible.

The acidity or alkalinity of the food vacuoles may be demonstrated by neutral red (1:50,000) which becomes a cherry-red color at the acid end and yellow toward the alkaline end of its color range, or by phenol-sulphonphthalein<sup>9</sup> which is pink in alkaline and yellow in acid conditions.

*Methods for Contractile Vacuoles.* To see the contractile vacuoles of *Paramecium* discharge outside of the body, place the animals into a very

<sup>7</sup> Cleveland, L. R. *Biol. Bull.*, 48: 282, 1925.

<sup>8</sup> Kirby, H. *Univ. Calif. Pub. in Zool.*, 29: 25, 1926.

<sup>9</sup> Shipley, P. G., and DeGaris, C. F. *Science*, 62: 266, 1925.



dense suspension of carbon particles in a small drop of water on a slip and cover with a cover glass.<sup>10</sup>

*Methods for Other Cytoplasmic Structures.* Stain mitochondria in Protozoa as in Metazoa with Janus green or Janus green B, diluting 1 to 10,000 or even 1 to 500,000.<sup>11</sup> Use neutral red in concentrations of 1 to 10,000 or weaker to give a diffuse cytoplasmic stain or to stain various structures selectively, for example Golgi bodies in gregarines<sup>12</sup> and sap-filled vacuoles of Dinoflagellates.<sup>13</sup>

*Basic dyes* appear to stain more readily intravital than do acid dyes. Ball<sup>14</sup> tested a number of dyes and gives the following list with the minimum concentration that would stain the cytoplasm of *Paramecium* and the toxicity as indicated by the percentage of animals dead at the end of one hour:

Dyes	Minimum Concentration That Will Stain <i>Paramecium</i>	Toxicity: Per Cent Dead in One Hour
Bismarck brown.....	1 to 150,000	0
Methylene blue.....	1 to 100,000	5
Methylene green.....	1 to 37,500	5
Neutral red.....	1 to 150,000	3
Toluidine blue.....	1 to 105,000	5
Basic fuchsin.....	1 to 25,000	30
Safranin.....	1 to 9,000	30
Aniline yellow.....	1 to 5,500	0
Methyl violet.....	1 to 500,000	20
Janus green B.....	1 to 180,000	40

Additional intravital dyes useful for Protozoa are brilliant cresyl blue (1 to 50,000), Nile blue (1 to 30,000) and rhodamine (1 to 20,000);<sup>15</sup> Azur 1, indulin, and Victoria blue in concentrations of 1 to 100,00 up to 1 to 200,000;<sup>16</sup> and a new stain, "spirsil," in concentrations of 1 to 32 or 1 to 64, as recommended by Varga.<sup>17</sup>

### III. Temporary Killing and Staining Methods

*Intravital dyes*, in strong concentrations, may often serve as both killing and staining agents. (1) Janus green B: add one drop of a 0.5

<sup>10</sup> Jennings, H. S. *Zool. Anz.*, 27: 656, 1904.

<sup>11</sup> Causey, D. *Trans. Am. Micr. Soc.*, 44: 156, 1925.

<sup>12</sup> Joyet-Lavergne, P. *Compt. Rend. Soc. de Biol.*, 94: 830, 1926.

<sup>13</sup> Dangeard, P. *Comp. Rend. Acad. de Sci.*, 177: 978, 1923.

<sup>14</sup> Ball, G. H. *Biol. Bull.* 52: 68, 1927.

<sup>15</sup> Becker, E. R. *Biol. Bull.*, 50: 235-238, 1926.

<sup>16</sup> Rumjantzew, A., and Kedrowsky, B. *Protoplasma*, 1: 189, 1926.

<sup>17</sup> Varga, L. *Ztschr. f. wiss. Mikr.*, 43: 338, 1926.

per cent stock solution to 10 to 20 drops of water (free-living forms) or salt solution (parasites) and either mix with the Protozoa on the slide or place it at one edge to be drawn under the cover by capillarity.<sup>18</sup>

(2) Use alcoholic solutions (saturated) of methylene blue, methyl green, gentian violet, safranin and other dyes diluted to various strengths in water in the same manner as the Janus green.<sup>19</sup>

*Writing fluids* may be also employed<sup>20</sup> for staining classroom material (Paramecia). Place a drop of culture on a slip and stir in a small quantity, "a dab or two" of Sanford's red ink with a toothpick. Put the cover glass in place and after four or five minutes add Waterman's ink from a fountain pen at one side of the cover glass. As the Paramecia come into contact with the blue ink they discharge their trichocysts, the cytoplasm becomes red, the cilia a "flame" color and the trichocysts blue. The nucleus usually stains also. While somewhat capricious, this method gives some very interesting results.

*Iodine* has long been used for killing Protozoa for quick examination.

(1) Lugol's solution (Potassium iodide, 6 gm., iodine, 4 gm., water, 100 c.c.) is commonly employed. Dilute this stock solution 1 to 5 or 1 to 10 with water and then mix with the organisms on the slide or add at one side of the cover glass. (2) Dilute a saturated solution of iodine in alcohol containing 3 per cent potassium iodide with water and use in the same way.<sup>21</sup>

*Osmic acid*, either in the form of vapor or in a 1 per cent aqueous solution is frequently used for treating Protozoa for temporary examination. (1) Invert the slide with the Protozoa on it over the mouth of a bottle or other vessel containing a 1 per cent solution of osmic acid and the vapor will kill the organisms in a few seconds, or, (2) mix a drop of the solution with the organisms on the slide. Causey<sup>22</sup> recommends the following for class use: centrifuge the culture; pour off the supernatant fluid and add a few drops of a 1 per cent solution of osmic acid so that the resulting mixture will be about a 1/2 per cent solution of osmic; add a little distilled water, and the material is ready for distribution to the class. If permanent mounts are desired, prolong the fixation for one-half to one hour.

*Copper Salts.* Treat Paramecium and other Protozoa with 1 per cent or 2 per cent aqueous solutions of copper sulphate, copper acetate, copper

<sup>18</sup> Hogue, M. J. *Stain Technology*, 1: 35, 1926.

<sup>19</sup> Hausman, L. A. *Am. Nat.*, 54: 333, 1920.

<sup>20</sup> Halter, C. R. *Science*, 60: 90, 1925.

<sup>21</sup> Hausman, L. A., *loc. cit.* p. 395.

<sup>22</sup> Causey, D. *Science*, 62: 113, 1925.

chloride or copper bichromate for temporary examination. Little or no distortion results.

*Alcohol.* To demonstrate the pellicle of *Paramecium* and other ciliates, apply a 35 per cent solution. The pellicle is raised up in blisters.

*Methods for Nuclei.* (1) Methyl green and acetic acid: use a 0.2 per cent to 0.5 per cent solution in 1 per cent acetic acid. Add at one side of the cover glass on the slide and it will diffuse under, killing the organisms that it may reach and staining the nuclei. (2) Methyl green in alcohol: use a 0.5 per cent solution in 50 per cent or 70 per cent alcohol. Employed as the acetic acid mixture this sometimes gives better results. (3) Aceto-carmin (p. 612): use as the methyl green solutions. It kills and provides a selective nuclear stain. Copper salts may be combined with the methyl green-acetic, as follows: to 100 c.c. of dist. water add copper acetate, 0.5 gm., copper chloride, 0.5 gm., glacial acetic acid, 1.0 c.c. and methyl green, 0.2 gm.

*Noland's*<sup>23</sup> stain for flagella and cilia. Moisten 20 mg. gentian violet with 1 c.c. water, then add sat. aq. sol. phenol in water, 80 c.c., 40 per cent formaldehyde, 20 c.c., and glycerin, 4 c.c. Mix a drop of this reagent with a drop of culture to be examined.

#### IV. Permanent Mounts

1. **General Section.** *a. General Directions.* The methods for making permanent mounts of any kind of Protozoa need to be adapted to the nature of the organisms, their habitat, and the special results desired. In general, it is not advisable to limit oneself to any one method of procedure. Different methods reveal different structures and where a full knowledge is desired, as many varied methods need to be employed as will reveal the greatest number of structural details.

*Surface Scums of Cultures.* If the culture bears a surface scum in which the Protozoa may be more or less entangled, drop a clean cover glass on the scum, then lift it up with forceps, keeping it horizontal, and a section of the scum will adhere to the under surface. Tilt the cover and drain off excess water on a piece of filter paper, then drop, scum side down, on the surface of the fixative which has been placed in a convenient container. Invert the cover in the fixative, bringing the scum side up, before it can drop to the bottom of the container and handle in this position until it is cleared and ready for mounting on a slip.

*Albumen Smears.* There are two ways of preparing albumen smears of free-living forms. (1) Smear a slip or cover with egg albumen, either fresh or in the form of Mayer's albumen fixative, and place a drop of the

<sup>23</sup> Noland, L. E. *Science*, 67: 535, 1928.

culture on this smear. Allow the culture fluid to evaporate until there is only a thin film. Then drop the smear, film side down, on the fixative and handle as indicated above. (2) Fix the organisms by pipetting them into the fixative in a test tube or bottle. When they have settled to the bottom decant or pipette off the fixative and pour in the washing fluid. By successively allowing the organisms to settle, or centrifuging, then decanting and pouring in the next fluid, carry the fixed specimens through graded alcohols to about 85 per cent alcohol, and then affix to a slip or cover as follows: Smear the slip or cover with a thin layer of albumen, then pipette a small drop of the alcohol containing the organisms on to the smeared surface. The alcohol spreads rapidly, distributing the contained specimens, and coagulates the albumen. Allow to evaporate a little, but before drying can occur, place the slide or cover in a dish of 90 per cent or higher strength of alcohol for further hardening of the albumen, after which the specimen is handled as a whole object, as indicated previously.

*Handling in Bulk.* Instead of making albumen smears, leave the organisms in the tubes or vials in which they are fixed through the entire series of steps up to clearing, concentrating at the bottom for each change of fluid either by sedimentation or by centrifuging. Mounting requires care. It is best to add thick balsam to the specimens in their container. A small drop of the thick balsam containing specimens is placed on a slip, then a drop of thinner balsam placed on this, and the cover glass applied. The thinner balsam will spread to the outer edge of the cover, and the organisms will stay nearer the middle. If the number of specimens is small it is best to transfer them in xylol to a watch glass and carry out the further steps under a dissecting binocular microscope. For the larger Protozoa it is well to support the cover with pieces of cover glass or fine glass rods.

*Fixing and Staining on a Slip.* Place a drop of water or culture containing the organisms on a slip and before the cover glass is applied, place a white cotton thread slightly longer than the width of the cover glass across the slip in one edge of the drop. Place an additional bit of thread on the other side of the drop to keep the cover level and put the cover in place. If, on examination, it is desired to fix and stain the organisms present, apply a blotter or other absorbent paper to the side of the preparation nearest the long piece of thread, to take up any excess water, then apply the fixative at the opposite side and draw under by withdrawing fluid with the blotter on the side with the thread. The entire series of processes of fixation, washing, staining, dehydration, clearing and mounting can be carried out without moving the cover. Clear

with clove oil or similar oil, after 95 per cent or absolute alcohol, and follow with xylol and then damar or balsam.<sup>24</sup>

*Washing, Dehydration, Etc.* After fixatives containing alcohol, wash out in 50 per cent or 70 per cent alcohol. Wash out aqueous fixatives with water. It is customary to put iodine in one of the washing fluids if the fixative has contained mercuric chloride. The writer finds that the iodine treatment of smears can be dispensed with if the washing can be prolonged for twenty-four hours or more, and the washing fluids changed several times. If the time of washing is to be short, the iodine treatment is advised, usually in the form of Lugol's solution added to the 70 per cent alcohol, 1 c.c. or 2 c.c. to 100 c.c. of the alcohol. Extra hardening in alcohols higher than 70 per cent is unnecessary with most of the fixatives mentioned below.

Downward steps from higher alcohols to water are usually accompanied by strong diffusion currents unless the changes are gradual. If it is inconvenient to employ the drop method (p. 254), smears may be passed through the following grades of alcohol: 70 per cent, to 50 per cent, to 30 per cent, to 10 per cent, to water, to stain. One to five minutes in each alcoholic solution is usually sufficient. After staining and washing, fewer steps are necessary for dehydration: e.g., water, to 30 per cent, to 70 per cent, to 95 per cent, to absolute alcohol or a clearing oil, to xylol, to balsam. Dioxan may be substituted for the alcohols and clearing oil. Damar may be substituted for Canada balsam and is preferred by many workers (p. 618).

*Mounting Individual Specimens.* Bowen's method.<sup>25</sup> Transfer the stained organisms from alcohol to ether-alcohol for one to several hours, then into thin collodion for twenty-four hours or less. Pour the collodion with the organisms into a flat dish and separate the individuals with a needle or probe. When partly hardened by evaporation, complete the hardening with chloroform vapor. Remove the thin sheet of collodion and cut into blocks, each containing a single specimen. These blocks can then be cleared in cedar wood oil and mounted in balsam.

Mueller's Method.<sup>26</sup> Transfer individual specimens from 95 per cent alcohol to a drop of euparal on a slip. Warm over an alcohol lamp or electric bulb and orient with a needle. Allow this to harden for a week out of reach of dust, then add a little fresh euparal and a cover glass. The hardened layer of euparal prevents any injury to the specimen in case the cover glass is broken. This method is especially advantageous for class room material.

<sup>24</sup> Tozer, E. J. *Roy Soc.*, p. 24, 1909.

<sup>25</sup> Bowen, W. K. *Trans. Am. Micr. Soc.*, 42: 156, 1923.

<sup>26</sup> Mueller, J. F. *Trans. Am. Micr. Soc.*, 45: 54, 1926.

*b. Fixation.* For any particular kind of Protozoa, the best fixatives can be determined only by experiment. In most cases a number of different fixatives need to be employed to bring out the different structural details. For example, in various species of *Trichomonas* it has been found that Janicki's parabasal body will not stain after Schaudinn's fixative, but will stain after chromic acid or osmic acid mixtures without acetic acid. Most of the standard cytological and histological fixatives can be used for Protozoa, but there are some which have come to be regarded as especially valuable for this group. Most of these are best used warm, and the fixation will be completed in a few minutes to an hour unless some special method like that for Golgi bodies is being used. When one is dealing with a piece of tissue, an hour or longer should be allowed.

*Sublimate Mixtures.* Schaudinn's fluid was originally made by taking 2 parts of a saturated aqueous solution of mercuric chloride and adding 1 part of absolute alcohol. Maier<sup>27</sup> took 200 c.c. of distilled water and added 1.2 gm. of sodium chloride, 10 gm. of mercuric bichloride, and 100 c.c. of absolute alcohol. Nowlin<sup>28</sup> makes Schaudinn's by adding 20 parts of absolute alcohol to 80 parts of a saturated solution of mercuric bichloride. Calkins<sup>29</sup> uses a saturated solution of mercuric chloride in 95 per cent alcohol. Most workers add from 1 to 5 parts of glacial acetic acid before using. Wenrich and Geiman<sup>30</sup> dilute the original Schaudinn's solution with an equal amount of distilled water, then add 2 to 5 per cent of glacial acetic acid. Another valuable modification is to use equal parts of original Schaudinn's solution and 2 per cent chromic acid, then add 5 per cent of glacial acetic acid. Since Schaudinn's fluid has become so generally employed for Protozoa it is recommended as a standard routine fixative, but for any complete investigation it needs to be supplemented by others.

*Other sublimate mixtures* which give results comparable to those given by Schaudinn's fluid are (1) sublimate-acetic (p. 557) and (2) Worcester's fluid (p. 557). These and Schaudinn's fluid are best used hot. In a series of tests made for the writer by Scott, the best fixation was obtained at 45°C.

*Picric Acid Mixtures.* Bouin's fluid (p. 560) is a very useful fixative for Protozoa, as are many of its modifications, such as Allen's B<sub>3</sub> (p. 561) and that of Wetzel<sup>31</sup> who takes 3 parts of a concentrated solution of

<sup>27</sup> Maier, H. N. *Arch. f. Protistenk.*, 2: 73, 1903.

<sup>28</sup> Nowlin, N. J. *Parasitol.*, 3: 143, 1917.

<sup>29</sup> Calkins, G. N., and Bowling, Rachel C. *Biol. Bull.*, 51: 389, 1926.

<sup>30</sup> Wenrich, D. H., and Geiman, Q. M. *Stain Technology*, 8: 158, 1933.

<sup>31</sup> Wetzel, A. *Arch. f. Protistenk.*, 51: 209, 1925.

picric acid to 1 part of formol and 1 part of acetic acid. Brazil's<sup>32</sup> alcoholic modification is as follows:

Picric acid .....	1.0 gm.
80 per cent alcohol.....	150.0 c.c.
Formol (full strength) .....	60.0 c.c.
Glacial acetic acid .....	15.0 c.c.

Picro-acetic is made up in various ways (p. 562). Dobell<sup>33</sup> added 1 part of glacial acetic acid to 3 parts of a saturated solution of picric acid in 90 per cent alcohol.

The writer finds Hollande's fluid useful especially for certain flagellates. It is made up as follows:

Picric acid .....	4.0 gm.
Copper acetate .....	2.5 gm.
Formol .....	10.0 c.c.
Glacial acetic acid.....	1.5 c.c.
Distilled water .....	100.0 c.c.

Hollande washed pieces of tissue in water, but the writer finds 50 per cent alcohol satisfactory for washing smears after this fixative, as with the other picric acid fixatives just given.

*Picro-mercuric Mixtures.* Yocum<sup>34</sup> used the following:

Bichloride of mercury.....	2.0 gm.
Picric acid .....	1.0 gm.
Alcohol, 95 per cent.....	110.0 c.c.
Ether .....	20.0 c.c.
Glacial acetic acid.....	20.0 c.c.
Formol .....	50.0 c.c.

*Chromic Acid, Osmic Acid, and Other Mixtures.* Chromo-acetic (p. 558), Champy's fluid (p. 560), Flemming's fluids (p. 559), Hermann's fluid (p. 560) and 1 per cent or 2 per cent osmic acid are all more or less used in the investigation of Protozoa. Some additional fixatives will be mentioned in the special section to follow.

*c. Staining.* The stains that one employs in any particular case need to be adapted (1) to the kind of animal to be stained, (2) to the fixative which has been used and (3) to the special purposes to be served. It is usually desirable to employ a number of different stains to gain a full knowledge of the structures of Protozoa.

*Iron Alum Hematoxylin and Modifications.* Heidenhain's method is fully described elsewhere (p. 613). It is probably the most generally

<sup>32</sup> Brazil, L. *Arch. de Zool. Expér. et gén.*, 34: 69, 1905.

<sup>33</sup> Dobell, C. *Arch. f. Protistenk.*, 34: 139, 1914.

<sup>34</sup> Yocum, H. B. *Univ. of Cal. Pub. in Zool.*, 18: 337, 1918.

useful single stain that may be employed for Protozoa, especially for the smaller forms and for sections of the larger ones. In general, the writer finds that the longer methods (twenty-four hours) give the best results, but briefer methods are often useful, e.g., mordant fifteen to thirty minutes, wash, and stain the same length of time; wash and destain as usual. If the fluids are kept at incubator temperature, these short periods will give better results.

*Regaud's Hematoxylin.* Dissolve 1 gm. of hematoxylin in a mixture of 80 c.c. of distilled water, 10 c.c. of glycerin and 10 c.c. of absolute alcohol. Stain as with Heidenhain's after mordanting with 4 per cent or 5 per cent iron alum.

*Kofoid and Swezy's Alcoholic Modification.*<sup>35</sup> Mordant ten minutes in a 4 per cent solution of iron alum diluted with 10 parts of 50 per cent alcohol; rinse in 50 per cent alcohol; stain ten minutes to one hour in a 0.5 per cent solution of hematoxylin diluted with ten parts of 70 per cent alcohol. Differentiate in the iron alum; wash in 50 per cent alcohol or in water for two hours; dehydrate, clear and mount.

*Dobell's alcoholic hematein modification.*<sup>36</sup> From 70 per cent alcohol, place slides or smears in a 1 per cent solution of iron alum in 70 per cent alcohol for ten minutes or longer. Rinse again in 70 per cent alcohol and place in a 1 per cent solution of hematein in 70 per cent alcohol for ten minutes or longer. Rinse again in 70 per cent alcohol and differentiate in the alcoholic iron alum solution or in 0.6 per cent hydrochloric acid in 70 per cent alcohol. Wash thoroughly in 70 per cent alcohol, dehydrate, and mount.

Other hematoxylin stains frequently used for Protozoa are Delafield's hematoxylin (p. 613), Ehrlich's hematoxylin, Mallory's iron chloride hematoxylin (p. 613) and Mayer's hemalum (p. 613).

*Counterstains* are frequently used after hematoxylin stains. Among those more commonly employed are: eosin, erythrosin, orange G, methyl green, light green, fast green FCF, acid fuchsin, Bordeaux red.

*The Carmine Stains.* Alum cochineal (p. 612), alum carmine (p. 612), borax carmine (p. 612), picro-carmine (p. 611), are also useful for staining Protozoa.

*Polychrome Stains.* Mallory's tricolor stain is frequently used, especially for sections. Sharp<sup>37</sup> used the following modification. After fixing in Zenker's solution and washing in water the preparation is treated thus:

Seconds

- |                                               |            |
|-----------------------------------------------|------------|
| (1) fuchsin S, 0.5 per cent aq. solution..... | forty-five |
| (2) distilled water .....                     | five       |

<sup>35</sup> Kofoid, C. A., and Swezy, O. *Proc. Am. Acad. Arts & Sc.*, 51: 289, 1915.

<sup>36</sup> Dobell, C. *Loc. cit.*, p. 534.

<sup>37</sup> Sharp, R. G. *Univ. Cal. Pub. in Zool.*, 13: 43, 1914.



Seconds

- |                                                 |       |
|-------------------------------------------------|-------|
| (3) phosphomolybdic acid, 1 per cent.....       | sixty |
| (4) fresh distilled water.....                  | five  |
| (5) aniline blue, orange G and oxalic acid..... | sixty |
| (6) distilled water .....                       | ten   |
| (7) 95 per cent alcohol.....                    | one   |
| (8) absolute alcohol .....                      | one   |
| (9) carbol-xylol .....                          | one   |
| (10) xylol to complete clearing, then mount.    |       |

*Giemsa's Stain, Wet Method.* After fixation (e. g., in Schaudinn's fluid) wash and treat for ten minutes with a 0.5 per cent solution of sodium thio-sulphate; wash in distilled water and stain several hours or overnight in Giemsa's stain (pp. 543, 588), diluting the stock stain 1 drop to 1 c.c. of neutralized distilled water or a buffer solution of pH 6.75. It is well to renew the stain after the first hour or two. The slide should be placed in the stain at an angle, with the smears or sections downward. Rinse in tap or buffered water, then dehydrate and clear by the following acetone-xylol steps: (1) pure acetone, 95 parts, xylol, 5 parts; (2) acetone, 70 parts, xylol, 30 parts; (3) acetone, 30 parts, xylol, 70 parts; (4) pure xylol; then mount in neutral balsam.<sup>38</sup>

*Short Giemsa Method* (p. 543). Fix wet smears in equal parts of methyl alcohol and ether; transfer to Giemsa's stain, 1 drop of stock stain to 15 c.c. of neutral or buffered distilled water for eight to ten minutes; wash in distilled water till pink color appears; dry in air and mount immediately in balsam.

*Mann's Methyl Blue-eosin Method.* The stain is prepared by mixing together:

1 per cent methyl blue in distilled water.....	35 c.c.
1 per cent eosin in distilled water.....	45 c.c.
Distilled water .....	100 c.c.

Arndt<sup>39</sup> modifies this by taking 3 parts of distilled water and adding 1 part of the 1 per cent methyl blue and 1 part of the 1 per cent eosin. (Note: *methyl blue* is used, not methylene blue.)

Shorter method of staining: transfer sections or smears from water to the stain for five to ten minutes, depending on the special needs, the thickness of the sections or smears, and the fixative used. Rinse in water, dehydrate, clear and mount.

The longer method, as employed by Dobell.<sup>40</sup> Stain four to twelve hours, wash in distilled water, then differentiate in a solution made by adding a few drops of saturated orange G to 100 c.c. of 70 per cent alcohol. Wash in water, dehydrate with graded alcohols, clear and mount.

*Alcoholic Eosin-methylene Blue Method.*<sup>41</sup> From absolute alcohol place in 1 per cent alcoholic eosin for one minute; wash in water; stain for one minute in

<sup>38</sup> Wenyon, C. M. Protozoology. Lond., 1926.

<sup>39</sup> Arndt, A. Arch. f. Protistenk., 49: 1, 1924.

<sup>40</sup> Dobell, C. The Amoebae living in Man. Lond., 1919.

<sup>41</sup> Lim, R. K. S. Quart. J. Micr. Sc., 63: 541, 1919.

1 per cent methylene blue in distilled water; wash in water; dry slide with a cloth or blotter, but leave tissue moist, differentiate quickly in absolute alcohol; clear in xylol or benzol and mount in balsam.

*Methods for Mitochondria* in the Protozoa are the same as the methods used for Metazoa (p. 265), or the following method of Causey<sup>42</sup> may be employed. Fix one to two hours in 1 per cent osmic acid or in osmic vapor and stain as follows: mordant in 5 per cent iron alum for thirty to forty minutes; rinse in distilled water, stain in Regaud's hematoxylin (p. 535) thirty to forty minutes; wash in water and destain in the 5 per cent iron alum, watching under the microscope. The time for destaining will vary with the size and species.

*Methods for Golgi Bodies.* King and Gatenby<sup>43</sup> demonstrated Gogli bodies in *Opalina* by the following method: Fix the host's rectum for twenty-four hours in Champy's fluid (p. 560); wash under the tap for twenty-four hours, then cut into small pieces and keep in 2 per cent osmic acid for four days at a temperature varying from 30° to 60°C. Wash the pieces in distilled water, dehydrate, imbed, and make thin sections. Mount the sections unstained. (For the method of Nassonov, see p. 550.)

*d. Sectioning.* The general principles for sectioning Protozoa are the same as those for sectioning tissues of the Metazoa (see Part 1). When Protozoa occur in the tissues of host animals, these tissues are handled just as other tissues are. Free-living Protozoa are usually sectioned en masse, i.e., a large number concentrated together, or as single individuals.

*Sectioning en masse.* The chief problem here is that of concentration. For this purpose, all steps up to clearing may be done by sedimentation or with the centrifuge. Clearing may be done with xylol, dioxan or with chloroform for paraffin imbedding. There are a number of methods of handling.

(1) Transfer the concentrated group of Protozoa with a pipette from xylol to a paper boat made by folding heavy paper into a small rectangular open box. Lower this box or boat into melted paraffin. Several changes of paraffin are advisable to insure the removal of all the xylol.

(2) Make a mold in hard paraffin with a brass rod which is square in cross section (Sharp).<sup>44</sup> Into this mold run the melted paraffin containing the animals and allow to cool. The hard paraffin of the mold may be tinted by sudan III to differentiate it better from the imbedding paraffin.

(3) Lefevre's watch glass may be used for imbedding. This has a straight-sided groove at the bottom. Pour the melted paraffin containing the Protozoa into the watch glass and sweep the organisms into the groove with a small brush or other agent. After hardening, trim off the projecting ridge of paraffin from the groove and section.

<sup>42</sup> Causey, D. *Loc. cit.*, p. 529.

<sup>43</sup> King, S. D., and Gatenby, J. Brontë. *Quart. J. Micr. Sc.*, 70: 217, 1926.

<sup>44</sup> Sharp, R. G. *Loc. cit.*, p. 535.

(4) Calkins<sup>45</sup> method. Fix ciliates in a test tube and add zooglea from an old *Paramecium* culture. Shake the tube to mingle the ciliates with the zooglea and again after each change of fluid through washing and clearing. There is thus obtained a well-matted mass which is stained in eosin to facilitate observation. Imbed in the same manner that a piece of tissue would be imbedded.

*Sectioning Individuals.* Single individuals may be handled in a watch crystal or round bottomed vial under the dissecting microscope. Staining with eosin helps in the recognition of the animal. The individual may be transferred from dish to dish with a pipette or may be left in one dish and the fluids changed with a pipette.

Imbed in the small white porcelain dishes commonly used for water colors. From xylol in the vial or watch crystal, transfer the object to xylol in the dish. Replace xylol by melted paraffin which is prevented from hardening by gently heating the stage of the microscope with a small lamp. Make several changes of paraffin. In the final paraffin, orient the object in the center of the dish with a fine glass needle with a bead tip. The dish is set in ice water to a depth that reaches to the top of the dish without overflowing it. As the paraffin cools from the bottom up, additional melted paraffin is added to prevent the depression that usually forms during cooling.<sup>46</sup>

Collodion imbedding is sometimes used (see Part 1) and is described by Scott<sup>47</sup> for sectioning *Balantidium*.

*Combined collodion and paraffin imbedding* may be accomplished as follows: From alcohol transfer to alcohol-ether, then to ether, using the centrifuge and decanting the supernatant fluids. To the organisms at the bottom of the tube, add a drop or two of liquid collodion. When evaporation has reduced this to the consistency of a thick syrup, the lenticular drop is taken out and placed in alcohol-chloroform, then pure chloroform, then imbedded in paraffin.<sup>48</sup>

**2. Special Section.** *a. Intestinal Protozoa. Fresh Material.* (For examination of living animals see pp. 526-527.) For examination of fresh material, dilute the intestinal contents or feces on a slip with a drop of saline solution and then treat with a 0.1 per cent to 0.5 per cent solution of eosin or with dilute iodine solution, or both together.<sup>49</sup> The eosin will stain nearly all the material in the preparation except the living Protozoa and their cysts, thus bringing them into relief against the pink background. The iodine solution (Lugol's solution diluted about 1 to 10

<sup>45</sup> Calkins, G. N. *J. Exper. Zool.*, 27: 293, 1919.

<sup>46</sup> Fry, H. J. *Anat. Record*, 34: 235, 1927.

<sup>47</sup> Scott, M. J. *J. Morphol. & Physiol.*, 49: 417, 1927.

<sup>48</sup> Collin, B. *Arch. de Zool. expér. et gén.*, 51: 1, 1912.

<sup>49</sup> Donaldson, R. *Lancet*, p. 571, 1917.

with water) will kill and stain the animals as well as stain the other objects, but will bring out the nuclei, the glycogen bodies in some of the protozoan cysts, and the motile organs of the vegetative ciliates and flagellates. Of these two methods, the iodine treatment is probably the more useful. McDaniels<sup>50</sup> emulsifies feces in a small drop of tap water, then adds an equal amount of a saturated solution of methylene blue in pure methyl alcohol. Nuclei of endamebae stand out as refractile or stained rings.

*Smears of intestinal protozoa* for fixation and staining may be made either on slips or cover glasses. An effort is made to get a thin, even film which is not so fluid that it will wash off on fixation, and not so viscid that it will dry before it can be fixed. The best results can be secured only after some experience. In most cases there is enough coagulable material in feces or intestinal contents to cause it to adhere readily to the slip or cover upon fixation. Dilution with weak salt solution (p. 526) is usually required, but occasionally such material is so fluid or so lacking in coagulable material that egg albumen or saliva will need to be added. The writer finds a small, curve-pointed forceps a convenient tool for making smears, and usually makes them on cover glasses, passing them through the reagents in Petri dishes, 8 to 12 in a dish. Feces or intestinal contents may also be fixed in bulk and for the large ciliates of ruminants this is usually advisable. Such material may be handled in one or another of the methods described in the general section (pp. 530-532).

**Fixation.** Choice of methods depends on the animals and the special purposes to be accomplished. Hot Schaudinn's fluid is most commonly employed but should always be supplemented by other fixatives. Zenker's fluid is sometimes used for subsequent staining with Mallory's triple stain or wet Giemsa stain. Chromic or osmic mixtures are useful for bringing out special structures, such as parabasal bodies (p. 541), mitochondria (p. 537), Golgi bodies (p. 537), etc.

**Staining.** Heidenhain's hematoxylin is the most generally useful stain except for the larger ciliates which should be stained with hemalum, Delafield's hematoxylin and similar stains or in Heidenhain's hematoxylin destained with a saturated solution of picric acid. In these cases, appropriate counterstains may be used. More detailed methods for the ciliates are given further on p. 548.

**Washing Cysts.** Mix thoroughly 5 to 10 gm. of feces in 100 to 200 c.c. of distilled water, sterile tap water or weak salt solution, then strain through several thicknesses of cheese cloth to remove coarser particles, and place the suspension in a tall vessel. The cysts and other heavier materials will fall to the bottom in two hours or more, when the super-

<sup>50</sup> McDaniels. *Science*, 79: 188, 1935.

natant fluid is poured off and fresh fluid is poured on and thoroughly stirred up. Allow to settle again, pour off the supernatant liquid, and pour on fresh fluid. Continue the process until the supernatant liquid is clear. The process may be hastened by using smaller quantities in a centrifuge. This method does not kill the cysts.

*Boeck's Concentration Method.* Place 1 gm. of stool in 30 c.c. of normal salt solution and thoroughly stir with a Hamilton-Beach "Cyclone" electric mixer for ten minutes. While still stirring, add 5 c.c. of ether. Cease stirring when the emulsion begins to foam the second time (i.e., 2 to 3 minutes longer). Quickly transfer to a separatory funnel and allow to stand for five to seven minutes. Draw off 15 c.c. of fluid at the bottom of the funnel and centrifuge for three minutes at 1600 revolutions per minute. Draw off the supernatant liquid and examine the residue.<sup>51</sup> The stirring may be done with a rod or woven-wire ladle held in the hand, but requires a much longer time.

*De Rivas' Concentration Method.* Place 1 or 2 gm. of feces in a test tube and add 5 to 10 c.c. of 5 per cent acetic acid. Closing the mouth of the tube with a rubber stopper or with the thumb covered by a sheet of rubber, shake the mixture vigorously till a more or less homogeneous suspension is produced. When feces are especially hard add a few glass beads to the material in the tube or break up the lumps with an applicator. Allow the suspension to stand for one-half to one minute during which time the acid appears to act on the fecal material, better preparing it for further treatment. It is well to filter the suspension through a double thickness of cheese cloth or through a finely woven copper wire mesh which can afterwards be cleaned by burning over a bunsen flame. Place about 5 c.c. of the suspension in an ordinary centrifuge tube and add an equal amount of ether. Close the tube and shake vigorously with the hand, holding the tube in a horizontal position. Next centrifuge for a few minutes. The material in the tube separates into four layers, the etherial extract at the top, the detritus plug near the middle, the acetic acid solution below and the sediment at the bottom. This sediment will contain the cysts of Protozoa and the eggs of worms, yeasts, and similar bodies.<sup>52</sup>

*Intestinal amebae* are best fixed in Schaudinn's fluid or other sublimate mixtures. An exception is *Dientamoeba fragilis* which, in the writer's experience, stains better after Bouin's or with Schaudinn's to which 10 to 20 per cent of glacial acetic acid has been added. The writer has also found that picromercuric will produce more "budding" on the cysts of certain amebae than will Schaudinn's. The most useful stain for

<sup>51</sup> Boeck, W. C. *Univ. Cal. Pub. in Zool.*, 18: 145, 1917.

<sup>52</sup> De Rivas, D. *Am. J. Trop. Med.*, 8: 63, 1928.

both vegetative stages and cysts is Heidenhain's hematoxylin, with or without counterstains.

*Intestinal Flagellates.* Fix in Schaudinn's, either full or one-half strength, or with chromic acid (see p. 533), Bouin's, or Hollande's mixtures for ordinary work. The writer has found the last one especially good for *Giardia*. For special structures like the parabasal body in Trichomonads, use chromic acid or chrom-osmic mixtures without acetic acid. Stain with Heidenhain's hematoxylin and counterstain with Bordeaux red, eosin, or light green and acid fuchsin. For staining the flagella see p. 547 or stain with Giemsa after making a thin dried smear.

*Intestinal Ciliates.* In most cases smears may be made in the usual way but the material from ruminant stomachs does not smear well unless albumen or saliva is added. For these ciliates, fixation in bulk in hot fixatives is recommended. These can then be treated as indicated in the general section (p. 530). Fix with the sublimate, picric acid, or chrom-osmic mixtures, and stain small forms with Heidenhain's hematoxylin followed by Bordeaux red, eosin, or other counterstain. Stain the larger ciliates with hemalum, Delafield's hematoxylin or similar stains and counterstain as with Heidenhain's. While Heidenhain's does not usually do so well with larger forms, good results can usually be obtained by destaining with a saturated solution of picric acid.

For finer details section the larger ciliates (pp. 548-550). Where the host is small, e.g., a frog, the intestine may be fixed and sectioned. In the case of Ruminants, fix the ciliates en masse and then handle by methods previously given (p. 531). Scott preferred imbedding *Balantidium* in collodion to paraffin for sectioning. Where the ciliates invade the tissues of the host (*Balantidium coli*) fix and section the tissues before staining.

*Intestinal Sporozoa: Gregarinida.* Gregarines are limited to invertebrate hosts but are not confined to the digestive tract of the hosts. However, since a majority do occur in the digestive tract, and all may be treated by the same general technique, they will be considered here as a unified taxonomic group. While gregarines are coelozoic through most of their development, they are frequently intracellular in the earlier stages, and even in later stages may remain attached to the host's tissues by special hold-fasts. Hence to get all stages and to see the relation of the parasites to the host's tissues, it is well to fix the parasitized organs and section them. Smears may be made in the usual way and are necessary for study of the entire animals.

Fix smears with sublimate or picric acid mixtures and stain smaller forms with Heidenhain's and larger forms with Delafield's or Ehrlich's

hematoxylin followed by eosin or other counterstain.<sup>53</sup> Berlin stained thin sections of monocysted gregarines with Hansen's hematoxylin, picro-fuchsin, Giemsa's stain and Heidenhain's hematoxylin, with counterstains of Bordeaux red, orange G, and safranin plus light green.

*Intestinal Sporozoa: Coccidia.* These are intracellular parasites but occur most commonly in the intestinal epithelium of their hosts and the oocysts are discharged into the lumen of the intestine to mingle with its contents and the feces. In many cases, the oocysts are highly resistant to chemical action and cannot be fixed and stained as can the other stages. In these cases developmental stages within the oocyst must be watched in the living condition. This may readily be done if the oocysts are kept for a few days in a 2-5 per cent solution of potassium bichromate in a Petri or other shallow dish. For the intracellular stages make smears, fix and stain like fecal smears, or fix the host's tissues, cut into pieces of convenient size, and section. These two methods are also advised for organs other than the digestive tract. Dobell<sup>54</sup> recommends Schaudinn's, Bouin's and Flemming's fixatives for smears, and Bouin's picro-acetic (p. 560) and sublimate-formol (3 parts of sat. aq. sol. of sublimate to 1 part of full strength formol) for tissues for sectioning. He cautions against allowing sea water to come into contact with the tissues of the marine hosts with which he worked. The writer finds that one-half strength Schaudinn's plus 5 per cent of glacial acetic acid is good for fixing coccidia in vertebrate intestines. For staining smears, Dobell recommends Delafield's hematoxylin, hemalum, picrocarmine, borax carmine, alum carmine and safranin (after Flemming's fixative). For sections he prefers his alcoholic hematein (p. 535), Heidenhain's, Mallory's iron chloride hematoxylin (p. 613) and others with counterstains of eosin, orange G, light green and Bordeaux red.

*b. Blood-inhabiting Protozoa.* (For methods of obtaining blood and of making blood smears, see p. 32.)

Thin films or smears are made in the usual way on slips or covers, an attempt being made to get an even film, so thin that the corpuscles will lie side by side but not overlie each other. The films are either dried at once, fixed before drying, or fixed in the cytological fixatives without drying.

*Fixation.* Blood stains made up in methyl alcohol act both as a fixative and stain. Aqueous stains require fixation before staining. The fixatives most generally employed are ethyl alcohol (95 per cent to 100 per cent), pure methyl alcohol, or a mixture of equal parts of alcohol and ether. Fix for five to thirty minutes, depending on the material and

<sup>53</sup> Watson, M. E. *Univ. Ill. Monographs*, 2: 211, 1916.

<sup>54</sup> Dobell, C. *Parasitology*, 17: 1, 1925.

stain to be used. Fixation with osmic acid vapor for five to thirty seconds before drying, or with formaldehyde vapor for one to two minutes is preferred by some, or the vapor fixation may be followed by alcohol before drying. (For fixation without drying, see p. 331.)

*Staining.* Before staining it is well to mark off the smear area on a slip with a wax pencil to prevent the stain from spreading over the entire surface. The aqueous stains which require previous fixation are Giemsa's, borax-methylene blue, and methylene blue-eosin.

*Giemsa's Stain.* (For preparation of the stock stain, see p. 328.) For staining, dilute the stock stain by adding 1 drop to each cubic centimeter of neutralized distilled water or buffer solution of pH 7.65 and add to the previously fixed smears. To avoid precipitates forming on the blood film, float cover smears on the surface of the stain or place the slide smears in a dish of the stain with the film side down. For the latter purpose a Petri dish may be used, the slip being supported at one end. Stain for ten to thirty minutes, depending on the material; wash thoroughly in tap water or buffer solution; dry and mount in neutral balsam or leave unmounted.

*Borax-methylene Blue* (Manson). Dissolve 2 gm. of methylene blue in a boiling solution of 5 gm. of borax in 100 c.c. of distilled water. Upon cooling, this stock is ready for use. De Rivas<sup>55</sup> dissolves 2 gm. of methylene blue in 10 c.c. of 95 per cent alcohol, then adds 100 c.c. of water and 5 gm. of borax. After shaking well he allows the mixture to stand over-night and then filters. For staining previously fixed smears, add 1 to 2 c.c. of the stock stain to 100 c.c. of distilled water. Stain one to several minutes, rinse thoroughly in water, dry and mount.

*Methylene blue-eosin* is prepared as follows:

Methylene blue, concentrated aq. solution.....	60 c.c.
Eosin, 0.5 per cent in 75 per cent alcohol.....	20 c.c.
Distilled water .....	20 c.c.
Potassium hydrate (20 per cent).....	12 drops

Stain five to ten minutes, dry, and mount in balsam.

*Wright's Blood Stain.* (For preparation, see p. 326.) Previous fixation is not required. Cover the blood film with the stock stain which is allowed to act as a fixative for one minute, then add distilled water, drop by drop, until a green scum begins to form on the surface. The amount of water added is usually about one-half the amount of stain, or not more than an equal amount. After dilution allow the stain to act for two to three minutes, then wash off thoroughly with distilled water, dry the film in the air and mount.

<sup>55</sup> De Rivas, D. Human Parasitology, Phila., 1920.



*Leishman's stain* may be purchased as a dry powder which is dissolved in pure methyl alcohol in the proportion of 0.15 gm. to 100 c.c. Allow this stain to act on a smear as a fixative for one-half to one minute, then dilute by two volumes of distilled water and allow to stain for two to five minutes longer. Wash off with distilled water, dry and mount.

*Russell's Combination Stain.* Place the blood smear (on a slip) for two minutes in undiluted Wright's stain (or other methyl alcohol blood stain); without washing place in a dish of tap water for three to five minutes; without washing transfer to dilute borax-methylene blue (1 c.c. of stock stain to 100 c.c. of water, made up fresh) for twenty to forty seconds. Wash quickly in tap water, dry and mount. This method combines the nuclear staining properties of Wright's stain with the cytoplasmic staining properties of borax-methylene blue.<sup>56</sup>

*Thick films* are often made for diagnoses, when parasites are suspected but are few in number, and dehemoglobinized before staining. Take a large drop of blood and spread it on a slip over an area 16 to 18 mm. across, or make a series of smaller drops on a slip without much spreading and allow to dry thoroughly. Gently move the slip about in a glass containing a 2 per cent solution of formalin to which has been added 1 per cent of glacial acetic acid. After the color has disappeared, treat the slip in the same way in a glass of water to remove all traces of acid. Wash gently in distilled water and stain with dilute Giemsa's stain (1 drop to 1 c.c. of neutralized distilled water) for twenty to thirty minutes, wash in distilled water and dry without heat or blotting paper (Stitt<sup>57</sup>). Other stains can, of course, be used.

*Wet Films.* Fix blood smears with the usual fixatives (Schaudinn's, etc.), then wash and stain either with polychrome stains (e.g., wet Giemsa, p. 536) or with hematoxylin stains, with eosin or other counterstains. Rees<sup>58</sup> recommends as follows: make smears, dry them and immerse in Schaudinn's solution without acetic; or, fix wet with formaldehyde fumes by placing in covered coplin jar containing a few cubic centimeters of full strength formalin, then treat with the Schaudinn's solution; wash as usual and stain with Heidenhain's hematoxylin or by the wet Giemsa method. Such preparations are thought to be better for cytological details.

*Relief Stains.* Instead of staining the parasites, the background may be dyed, leaving the parasites clear on a colored background. This method is more especially useful for the plasma-inhabiting forms, such as the trypanosomes. (1) Mix a drop of fresh blood on a slip with a drop of a thick suspension of India ink and make a smear of this in the usual way, then dry and mount in balsam or examine with immersion oil without a cover glass. (2) Employ in the same way a saturated solution

<sup>56</sup> Russell, F. F. *J. Am. Med. Ass.*, 64: 2131, 1915.

<sup>57</sup> Stitt, E. R. *Practical Bacteriology, Blood Work and Animal Parasitology*. Ed. 7, Phila., 1923.

<sup>58</sup> Rees, C. W. *Science*, 71: 134, 1930.

of opal blue and similar stains (Bresslau<sup>59</sup>) or nigrosin (Coles<sup>60</sup>) or a mixture of 3 parts of a saturated solution of china blue to 1 part of a saturated solution of cyanosin which has been allowed to stand one day after sterilization by boiling.<sup>61</sup>

*Hemoflagellates.* The methods given in the preceding paragraphs are applicable not only to flagellates found in the blood of their hosts, but also to related flagellates (Crithidia, Herpetomonas, Cryptobia), found in the digestive tracts of invertebrate hosts and to tissue-inhabiting hemoflagellates such as Leishmania. In these cases either make dried smears and stain like blood films, or fix with the usual fixatives, and stain with cytological stains. The latter choice is usually made for the study of cytological details. Tissues may be sectioned and the sections stained by the usual methods. One additional method may be mentioned.

*Gold chloride method* for myonemes of trypanosomes. Fix wet smears in Schaudinn's fluid. After washing, leave for twenty-four hours in 1 per cent gold chloride. Wash in water, then place in 1 per cent formic acid and set in strong light until a purple-red color appears. If direct sunlight is available, two to three hours should suffice. Dehydrate in graded alcohols, clear in xylol and mount in cedar oil.<sup>62</sup>

*Hemosporidia.* In addition to malarial organisms, other blood-inhabiting Sporozoa, including hemogregarines and piroplasms may be handled by the methods previously given. In most cases it is desirable to supplement dried film methods by wet fixation and cytological staining.

*c. Sarcodina.* Free-living Sarcodina (for endozoic amebae, see p. 540) are so different among themselves in size and organization that it is difficult to formulate general rules for their treatment. For cytological details they are usually fixed with sublimate, picric acid or chrom-osmic mixtures followed by staining of the smaller species entire with Heidenhain's, Delafield's hematoxylin, or hemalum, with counterstains, or with wet Giemsa's stain, Mallory's triple stain or Mann's methyl blue-eosin. Larger kinds may be stained entire with hemalum or Delafield's hematoxylin or sectioned and stained with Heidenhain's hematoxylin or the other stains just given.

*Amebae.* Dobell<sup>63</sup> (p. 542) prefers staining with his alcoholic hematein (p. 535) after picric acid fixatives. Wiener<sup>64</sup> proceeds as follows:

<sup>59</sup> Bresslau, E. *Arch. f. Protistenk.*, 63: 467, 1921.

<sup>60</sup> Coles, A. C. *Watson's Microscope Rec.*, p. 23, 1927.

<sup>61</sup> Eisenberg, *Zentralbl. Bact., Ref.*, 54: 145, 1912.

<sup>62</sup> Ogawa, M. *Arch. f. Protistenk.*, 29: 248, 1913.

<sup>63</sup> Dobell, C. *Loc. cit.*, p. 542.

<sup>64</sup> Wiener, E. *Arch. f. Protistenk.*, 39: 105, 1918.

Spread culture amebae on a slide and allow them to dry. Fix with alcohol, then place for five minutes in 1 per cent iodine; rinse in water and let drain. Stain one and one-half minutes in Loeffler's methylene blue (p. 137); rinse in water and drain, then one minute in a concentrated solution of eosin diluted with 3 volumes of water. Rinse the slide and mount in balsam. According to Beers,<sup>65</sup> rhizopods (*Amoeba* and *Arcella*) may be attached to a slip by allowing a drop of culture to remain on the slip for one-half hour; pour off the water and flood with cold Schaudinn's solution. The animals will adhere to the slip which may now be washed and stained as for any smear on a slip.

*Foraminifera*. Separate living Foraminifera from sand and debris by stirring up the collection mass in water and allowing it to settle for a brief time. The organisms will settle to the bottom of the container with other heavier material, but the finer particles will remain suspended and can be decanted off (Carpenter).<sup>66</sup>

Dead Shells. Carefully dry the collection mass in an oven, stirring several times, then sift into water. The air-filled shells should float on the surface of the water and the sand go to the bottom (Carpenter). Or, thoroughly stir the collection mass into a vessel of water until everything that will sink has gone to the bottom. Place a teaspoonful at a time of the wet material in a saucer or similar dish, spread out and cover with water to a depth of about 5 mm. Rotate the dish carefully and the Foraminifera shells will come to the surface of the sand and can be removed with a ball pipette (Vorce).<sup>67</sup>

Thin sections of Foraminifera shells for the study of the finer details are prepared by grinding. Usually the shells are attached to a glass slip with hard balsam and ground on appropriate grinders much as teeth are ground (p. 353).

For cytological details prepare by fixing with the usual fixatives, decalcify with weak hydrochloric acid in 60 per cent alcohol<sup>68</sup> and stain entire with borax carmine, hemalum, Delafield's, etc., or section and stain with Heidenhain's with counterstains.

*Heliozoa*. Fix with the usual fixatives and stain smaller forms entire with Heidenhain's or Delafield's hematoxylin, carmalum, borax carmine, or alum carmine.<sup>69</sup> Stain larger forms like *Actinosphaerium* entire with hemalum and similar stains or section and stain with Heidenhain's hematoxylin with counterstains. Axopodia are best demonstrated by silver impregnation methods<sup>70</sup> (p. 275).

*Radiolaria*. Separate skeletons from recent deposits by washing with

<sup>65</sup> Beers, C. D. *Science*, 72: 122, 1930.

<sup>66</sup> Carpenter, W. B. *The Microscope and its Revelations*. Ed. 8, Lond., 1901.

<sup>67</sup> Vorce, C. M. *Am. Month. Micr.*, J. 1: 24, 1880.

<sup>68</sup> Schaudinn, F. *Ztschr. f. wiss. Zool.*, 59: 191, 1895.

<sup>69</sup> Zuelzer, M. *Arch. f. Protistenk.*, 17: 135, 1909.

<sup>70</sup> Rumjantzew, A., and Wermel, E. *Arch. f. Protistenk.*, 52: 217, 1925.

a gentle stream of water through sieves of various grades. The smallest skeletons will go through all the sieves, but will fall to the bottom of the receptacle and the finer suspended particles of dirt, etc., can be decanted off.<sup>71</sup> If Foraminifera shells are to be removed also, treat with dilute nitric acid.

Fossilized deposits require repeated treatment with hot soda solutions alternated with drying. This treatment may destroy the more delicate skeletons (Earland).

For cytological details, fix skeletonless Radiolaria, or those with loose spicules, with sublimate mixtures, Flemming's or Hermann's fluids,<sup>72</sup> then section and stain with Heidenhain's or Kleinenberg's hematoxylin, etc.<sup>73</sup> Stain animals with skeletons entire with hemalum or the carmine stains.

*d. Mastigophora.* Animal flagellates can be treated for the most part by the methods described for the intestinal species (p. 541). Hemoflagellates are treated in a separate section (p. 545). One of the difficulties in the handling of flagellates is to get a good flagellar stain. For many forms the flagella can be demonstrated by making dried smears and staining as for blood flagellates or by using the relief staining method (pp. 544, 549). Such preparations do not, as a rule, give good results for the remainder of the cell. Few methods will stain flagella and the other components of the cell equally well; the following gives fair results.

*Hollande's<sup>74</sup> Method for Demonstrating Flagella.* Fix tissues or smears in the picro-formol-copper-acetate-acetic mixture already given (p. 534) for three to five days and wash in water for twenty-four to forty-eight hours. Sections (or smears) are then stained with Heidenhain's in the usual way, using 3 per cent iron alum as a mordant. After the usual differentiation, place in 1 per cent aqueous eosin for two to twenty-four hours; wash several seconds in distilled water to remove the excess of eosin; place in a 1 per cent aqueous solution of phosphomolybdic acid for five to ten minutes; wash with tap water thirty seconds; place in 0.2 per cent to 0.5 per cent aqueous solution of light green for several seconds to one minute, according to the time in the eosin and phosphomolybdic acid; dehydrate rapidly in graded alcohols; differentiate in 96 per cent alcohol for one to ten minutes, examining from time to time under the microscope. Arrest the differentiation by placing in pure amyl alcohol in which dehydration is completed. A second bath in amyl alcohol is advisable. Clear by passing first into equal parts of amyl alcohol and xylol, then into xylol, and mount.

*Chrysomonadina.* Fix in a mixture of 2 volumes of a saturated aqueous solution of boric acid and 3 volumes of a saturated solution of mercuric chloride

<sup>71</sup> Earland, A. *Nature*, p. 110, 1922.

<sup>72</sup> Huth, W. *Arch. f. Protistenk.*, 30: 1, 1913.

<sup>73</sup> Bogert, A. *Zool. Jahrb. Abt. f. Anat. u. Ontog.*, 14: 203, 1900.

<sup>74</sup> Hollande, A. C. *Arch. de Zool. expér. et. gén.*, 59: 75, 1920.

for three hours and wash in water<sup>75</sup> or fix with osmic vapor or Schaudinn's fluid,<sup>76</sup> and stain with Bordeaux red and Heidenhain's or wet Giesma, or Mallory's triple stain.

*Volvocidae.* Fix with Schaudinn's or Flemming's and stain with Heidenhain's with counterstains or with safranin and light green.<sup>77</sup>

*Euglenoids.* For flagellum and gullet fix one to three hours in Altmann's fixative (p. 266) and stain with Regaud's hematoxylin (p. 535). For blepharoplasts, cytoplasmic rhizoplasts and nucleus, fix in hot Schaudinn's fluid two to ten minutes and stain with Heidenhain's (twenty-four hours) preceded by immersion for twenty-four to forty-eight hours in a 0.2 per cent solution of Bordeaux red.<sup>78</sup> These methods are very useful for other flagellates.

*Dinoflagellates.* Fix in Flemming's fluid. Stain entire organisms with Delafield's or picrocarmine, sections with Heidenhain's<sup>79</sup> or fix with Schaudinn's and stain with Bordeaux red-Heidenhain's or borax carmine or eosin and methylene blue.<sup>80</sup>

*e. Infusoria.* The choice of technique will depend on the nature of the material and the special results desired. For most purposes, Zenker's, Schaudinn's, Bouin's, Flemming's, picro-sulphuric, etc., are adequate. For smaller ciliates, Heidenhain's hematoxylin, with appropriate counterstains such as eosin, fast green, or especially prestaining with Bordeaux red will give an adequate picture of the structural organization. Heidenhain's is not so well adapted to the larger ciliates, although good results can usually be obtained by using Tuan's<sup>81</sup> method of destaining with a saturated aqueous solution of picric acid; thorough washing in water should be followed by passing through a weak solution of ammonia. Sharp<sup>82</sup> and others have claimed success with the following modification:

*Sharp's Modification of Heidenhain's for Larger Ciliates.* Mordant twenty-four to thirty-six hours in a 1 per cent solution of iron alum; wash with distilled water; stain twenty-four hours with 0.3 per cent aqueous hematoxylin; wash in tap water and differentiate in the 1 per cent iron alum. Wash, dehydrate, etc., as usual.

For whole mounts of larger ciliates, stain with hemalum, Delafield's hematoxylin, or similar stains, counterstain with eosin, light green, orange G or Bordeaux red.

Sections of ciliates are necessary for finer details. Stain thin sections  $2\mu$  to

<sup>75</sup> Zacharias, O. *Zool. Anz.*, 22: 70, 1899.

<sup>76</sup> Doflein, F. *Arch. f. Protistenk.*, 44: 149, 1922.

<sup>77</sup> Hartmann, M. *Arch. f. Protistenk.*, 39: 1, 1918.

<sup>78</sup> Hall, R. P., and Powell, W. N. *Trans. Am. Micr. Soc.*, 45: 256, 1926.

<sup>79</sup> Lauterborn, R. *Ztschr. f. wiss. Zool.*, 59: 167, 1895.

<sup>80</sup> Hall, R. P. *Univ. Cal. Pub. in Zool.*, 28: 29, 1925.

<sup>81</sup> Tuan, H. C. *Stain Technology*, 5: 135, 1930.

<sup>82</sup> Sharp, R. G., *Loc. cit.*, p. 535.

4 $\mu$  thick, in Heidenhain's hematoxylin with a counterstain, or Mallory's triple stain (see Sharp's modification, p. 535) will give good results.

*Relief Staining.* Surface markings, ciliary rows, etc., are seen in sections, but whole mounts may be prepared by the methods of Bresslau, Coles and Klein. Among the stains used are (1) a mixture of 3 parts of a saturated solution of china blue with 1 part of a saturated solution of cyanosin (Bresslau's "cyanochin"), (2) a mixture made by adding 4 to 6 drops of a 6.5 per cent aqueous solution of phlorenrhodamin to 1 c.c. of a 10 per cent aqueous solution of opal blue (Bresslau, p. 545), (3) a saturated aqueous solution of nigrosin (Coles, p. 545). Place a drop of water or culture containing the ciliates in the middle of a slip and near it a drop of the stain. Thoroughly mix the two, spread in a thin layer, and allow to dry in the air. Mount in balsam or examine without a cover. The surface depressions of the animals retain the stain thus outlining the positions of the cilia.

*Silver Impregnation Method.*<sup>88</sup> Spread a drop of culture containing the ciliates in a thin layer on a slip or cover glass and allow it to dry in the air. Place for six to eight minutes in a 2 per cent solution of silver nitrate in distilled water, rinse well in distilled water contained in a white porcelain (or other) dish and set in strong daylight. Reduction follows in from four to ten hours, according to the intensity of the light. When the silver nitrate has become sufficiently reduced (test by examination with the microscope), rinse well, dry and mount in balsam. A network of "silver lines" becomes demonstrated by reducing the silver nitrate.

Sharp<sup>84</sup> studied the surface markings of *Diplodinium* by fixing in 4 per cent formalin and mounting unstained in styrax.

*Cilia.* For fresh-killed animals, fix with osmic acid or osmic vapor and then treat with 5 per cent soda solution (Maier<sup>85</sup>), or with bromine vapor.<sup>86</sup> The finer structures can best be studied in thin sections. Fix in Schaudinn's or Flemming's (Maier) or bibromate-acetic (potassium bibromate, 3 parts; glacial acetic acid, 5 parts; water, 100 parts), (Wetzel, p. 533), or fix in the regular fixatives followed by treatment with brome-bromine water (1 drop of pure bromine in 2 to 3 c.c. of strong bromine water) for ten to twelve hours, then wash with distilled water. Section and stain with Bordeaux red-Heidenhain, Mallory's triple, or other combination staining methods.

*Trichocysts.* Stain with Heidenhain's, especially in sections, after strong fixatives such as Schaudinn's fluid. To cause trichocysts to discharge in fresh preparations use weak concentrations of such acids as acetic, tannic, picric, osmic, chromic, etc., or bromine.

*Trichites.* Prepare these pharyngeal rods for study by the methods given above for cilia. MacDougall<sup>87</sup> found that they were not affected by dilute acids or dilute alkalis but were digested by artificial gastric juice (0.2 per cent HCl and 1 per cent pepsin in 100 c.c. of water), which indicated a protein composition.

<sup>88</sup> Klein, B. M. *Zool. Anz.*, 67: 160, 1926.

<sup>84</sup> Sharp, R. G. *Loc. cit.*, p. 535.

<sup>85</sup> Maier, H. N. *Loc. cit.*, p. 533.

<sup>86</sup> Khainsky, A. *Arch. f. Protistenk.*, 21: 1, 1910.

<sup>87</sup> MacDougall, M. S. *Quart. J. Micr. Sc.*, 69: 361, 1925.

*Contractile Vacuoles.* Stain in a fresh condition with alizarin blue (Grüb-ler's). They show well in sections prepared by the methods for demonstrating Golgi bodies. Fix with Altmann's fixative (p. 266) and stain with Heidenhain's hematoxylin,<sup>88</sup> or use the method of Nassonov.<sup>89</sup> Fix for twenty-four hours in either of the following:

- |                                           |         |
|-------------------------------------------|---------|
| (1) Potassium bichromate, 3 per cent..... | 2 parts |
| Chromic acid, 1 per cent.....             | 2 parts |
| Osmic acid, 2 per cent.....               | 1 part  |
|                                           |         |
| (2) Potassium bichromate, 6 per cent..... | 1 part  |
| Chromic acid, 1 per cent.....             | 1 part  |
| Osmic acid, 2 per cent.....               | 1 part  |

Wash thoroughly in distilled water and place in 2 per cent osmic acid for three to four days at a temperature of 35°C. Wash thoroughly in water, and dehydrate in graded alcohols. Small species can be mounted whole, but larger ones, e. g., *Paramecium*, need to be sectioned (paraffin). Subsequent staining is unnecessary. From the selective action of the contractile vacuole apparatus on osmic acid, Nassonov concluded that it is homologous with the Golgi apparatus of Metazoa.

*Neuromotor Apparatus.* Fix in Schaudinn's, Zenker's, Flemming's, etc. Stain in Heidenhain's or Mallory's triple (see Sharp's modification, p. 535). These structures are usually stained well by Heidenhain's hematoxylin in thin sections. The writer finds that the system of fibers described by Rees<sup>90</sup> can be demonstrated in *Paramecium* by fixation with Schaudinn's fluid at 75°C., and staining entire with hemalum or Delafield's hematoxylin and mounting whole.

*f. Sporozoa.* Myxosporidia. (Other sporozoa are considered elsewhere: Gregarinida, p. 547; Coccidia, p. 542; Haemosporidia, p. 546). Myxosporidia are either histozoic or celozoic. For those in tissues either smear on slips or covers, then fix and stain like intestinal smears (p. 539), or fix the containing tissues, imbed and section. Stain the sections with the hematoxylin or polychrome stains. Remove coelozoic forms from the host and make smears of them, or fix the containing organs and section.

Fix with Schaudinn's, Bouin's, Flemming's and Hermann's fluids, or Worcester's fluid.<sup>91</sup> Stain with Heidenhain's or Delafield's with or without counterstains, or with Mallory's triple, or wet Giemsa, for both smears and sections.<sup>92</sup>

<sup>88</sup> Young, R. A. *Science*, 60: 244, 1924.

<sup>89</sup> Nassonov, D. *Arch. f. Mikr. Anat. u. Entwickl.*, 103: 437, 1924.

<sup>90</sup> Rees, C. W. *Univ. Cal. Pub. in Zool.*, 20: 333, 1922.

<sup>91</sup> Davis, H. S. *J. Morphol.*, 37: 425, 1923.

<sup>92</sup> Awerinzew, S. *Arch. f. Protistenk.*, 14: 74, 1909.

Mavor, J. W. *Proc. Am. Acad. Arts & Sc.*, 51: 551, 1916.

Shrinkage of spores. Kudo<sup>93</sup> tried a number of common fixatives and found that they caused a decrease of about 14 per cent in the sutural diameter of the mature spores of *Leptotheca ohlmacheri*. In making measurements of spores, therefore, the fresh spores should be measured, or allowance be made for shrinkage.

*Microsporidia* may be handled by the methods given above for Myxosporidia. For the identification of spores (1) exert pressure on the cover glass over freshly teased out spores or add hydrogen peroxide to cause the extrusion of the filament. Such extrusion will identify the spores as distinct from bodies of similar size and shape, such as yeast cells. Oshima<sup>94</sup> adds hydrogen peroxide to a suspension of spores in a 5 to 10 per cent solution of NaCl to cause slow extrusion of filaments. (2) Stain with Ziehl's fuchsin, followed by decolorization with a solution of weak sulfuric acid. Yeast cells are decolorized while spores of *Microsporidia* remain red.<sup>95</sup>

*Sarcosporidia* are found usually as "Miescher's tubes" in the muscles of vertebrate hosts (browsing and grazing animals, mostly). Fix pieces of the tissues and section, or make smears of the tissues. Fix with Bouin's or Flemming's solutions or sublimate-alcohol-acetic. (Saturated solution of sublimate, 75 c.c., absolute alcohol, 25 c.c., and glacial acetic, 15 c.c.), and stain with hematein, hemalum, Mann's methyl blue-eosin, or Heidenhain's hematoxylin followed by eosin and picro-indigo-carmin.<sup>96</sup>

<sup>93</sup> Kudo, R. *Trans. Am. Micr. Soc.*, 40: 161, 1921.

<sup>94</sup> Oshima, K. *Annot. Zool. Japon.*, 40: 235, 1927.

<sup>95</sup> Kudo, R. *Ill. Biol. Monographs* 9, Nos. 2 & 3, 1924.

<sup>96</sup> Alexeieff, A. *Arch. de zool. expér. et gén.*, 51: 521, 1913.



## CHAPTER IX

### FIXATION AND FIXATIVES

C. E. McCLUNG AND EZRA ALLEN

CHARACTER OF AGENT USED 552. Physical agents 552. Chemical agents 552. DIFFERENTIAL EFFECTS OF FIXATIVES 562. Selective action 562. Penetration 563. Influence of physical condition 564. METHODS OF APPLICATION 565. By immersion 565. Fixation by injection 566. RELATION TO AFTER PROCESSES 569. Washing 569. Staining 570.

#### A. CHARACTER OF AGENT USED

The agents used for the purpose of fixation may be divided generally into physical and chemical, the latter being much the more common.

##### I. Physical Agents

The only physical agents employed are desiccation and heat.

1. **Desiccation.** Only infrequently can desiccation be applied, as, for instance, in the smear method recommended by Foot and Strobell (p. 264). Here the fresh tissue is drawn rapidly and steadily across the surface of the glass slip, leaving a thin film behind. Without further treatment the material is then stained and mounted. Not many tissues will return good results with this treatment.

2. **Heat.** More commonly in smears the protoplasm is coagulated and quite rapidly freed from water by passing it through an alcohol flame. Heat may also be used for large pieces of tissue or even whole animals. In this event the material is placed in water which is rapidly brought near to the boiling point, at which temperature the protoplasm is completely coagulated. Such a method is particularly applicable in the case of insect material. It is only infrequently employed for vertebrate tissues. Except for thin films, the action of a physical agent is less satisfactory than that of a chemical one.

##### II. Chemical Agents

For the convenience of discussion in part, and also because of distinctive peculiarities, these may be subdivided into purely liquid substances, solutions of solids, combinations of agents, and vapors.

1. **Purely Liquid Substances.** *a. Examples.* Of the first group may be mentioned:

*Acetic Acid.* This is one of the most valuable and generally used of reagents, but only infrequently is it employed alone. However, in some instances where it is desired to fix strongly contracting organisms very rapidly, acetic acid is most useful. For best results under these circumstances it may be employed warm. When thus used the action is very rapid and should be terminated within ten or fifteen minutes. The excess of fixing fluid is washed out with 30 to 50 per cent alcohol and the material preserved in 70 per cent. The general action of acetic acid is to swell the constituents of the cells, and because of this property it is generally combined with some substances which have an antagonistic or shrinking effect. The composition of fixing fluids, in which acetic acid is involved, is therefore a matter of experiment to determine what percentage of the swelling agent should be combined with others which have an opposite action.

For formulas in which acetic acid is combined with other agents, consult the various groups of fixatives.

Aside from the use of absolute acetic acid as indicated, dilutions from 1 to 5 per cent are most useful. It is in proportions like these that acetic acid is combined with other fixing materials. In all dilutions the penetration is good. It is also very valuable because of the strong differentiation which it establishes.

*Alcohol.* Alcohol, used alone, is employed either as absolute alcohol, or in a strength of approximately 30 per cent. The effective action of absolute alcohol is said to be due to its rapidity of operation, which thereby prevents the distortion of materials caused by rapid combination with their contained water. On the contrary, 30 per cent alcohol is a fairly weak fixative and if applied too long may even act as a disassociating agent for some tissues. For this reason, after fixation of not over twenty-four hours, the tissue should be run up to 70 per cent.

Alcohol is used in combination with other fixing agents especially in the form of absolute alcohol. For these combinations consult the various groups of fixatives.

*Chloroform.* Chloroform alone is rarely or never employed as a fixative. It finds its chief application in the combinations of Carnoy and of Carnoy-Lebrun (p. 558).

*Formic Acid.* Formic acid may be used in much the same manner as acetic acid in fixatives but has been found less satisfactory and is not generally employed. An example of the use of formic acid is afforded in the formula by Rable, which is as follows:

0.33 per cent chromic acid solution..... 200 c.c.  
Formic acid ..... 5 drops  
Fix for twelve to twenty-four hours. Wash in water.

*Formol.* This substance appears on the market as a 40 per cent aqueous solution of formaldehyde under the trade names of formol, formalin and formolose. It is best to use only the chemically pure solution. The formulas are sometimes ambiguous in not indicating whether it is the strength of the formaldehyde or the percentage of the formol that is meant. For convenience it seems better to indicate the percentage of the solution rather than of the gas. Solutions decompose after a time, forming paraformaldehyde, a white substance. Owing also to the transformation of formaldehyde into formic acid there is often an acid reaction of the solutions. This may or may not be an advantage, but the condition should be recognized. If a neutral solution is required, the addition of an alkali like sodium or magnesium carbonate or even of lithium carbonate, will serve. Formalin may be used alone and gives a fairly good fixation. It is very convenient because no extensive washing is required before sectioning. It has a high degree of penetration and is therefore applicable to large objects. It may be followed by most stains. Formalin is most commonly used in clinical work, when, because of its convenience, rapidity, and general applicability, it lends itself to the conditions of rapid routine work. It may well be questioned, however, whether or not the added precision of the picro-formol-acetic mixtures would not repay the slightly greater time involved in their use.

In using formalin with other substances it should be remembered that formaldehyde is a very powerful reducing agent and therefore rapidly alters the nature of combinations into which chromic acid or other such substances enter. Used alone it is best employed in strengths of 4 to 10 per cent. Fix for several days for best results. Formalin also is a good hardening agent.

*Nitric Acid.* Nitric acid is not used to any great extent alone as a fixative, but may be employed in a strength of 2 to 5 per cent, and is reported to be valuable because it makes certain tissues brittle.

*b. Advantages.* These simple fluids have some striking advantages which make them particularly applicable to certain cases. As a rule they penetrate well and rapidly. They leave no deposits in the tissues and they require little or no washing. In cases where rapid results are required there are very obvious advantages. For general routine work various dilutions of formol are much used, as they are also for very resistant objects such as the ova of certain worms and insects.

*c. Disadvantages.* The very qualities which make these liquids advantageous in some circumstances make them inapplicable in others.

Thus, their rapid and vigorous penetration may be injurious to very delicate structures, although under some circumstances they have the opposite effect.

**2. Solutions of Solids.** These solutions, employed as fixatives, are mostly aqueous in character. Chemically they are either salts or acids. The salts are commonly chlorides of some heavy minerals like gold, platinum and mercury, chromates of the haloid group, and the tetroxide of osmium. The acids most used are picric and chromic. Solutions of solids alone, without the presence of acetic acid or formol, are rarely employed as fixing agents.

*a. Classes.* Picric acid is not often used in simple solution, since it shrinks strongly. When combined with other substances it is in strong concentrations. Dilute solutions of picric acid macerate. Since it forms only a weak association with the tissues, washing out should be done with alcohol of at least 70 per cent to avoid maceration. When combined with formalin, however, this precaution is not necessary. Since picric acid is also used as a stain it need not always be completely washed out of the tissue if the color is not objectionable. To facilitate the removal of the picric acid, lithium carbonate may be added to the 70 per cent alcohol. Picric acid penetrates well and may be followed by most stains. The simplest method of handling picric acid is to make it up into a saturated solution in distilled water, and it is in this strength that it enters into most combinations.

*Chromic Acid* ( $\text{H}_2\text{CrO}_4$ ), a substance produced when chromic anhydride ( $\text{CrO}_3$ ) unites with water, is rarely used alone as a fixative, but forms a very valuable constituent of numerous mixtures. Combined with acetic acid it is a common fixative of plant tissues, and with the addition of osmic acid appears in Flemming's mixture as one of the most valuable of our cytological fixatives.

The crystals of chromic anhydride are very deliquescent and it is customary to keep the material in aqueous solutions of a strength of 1 or 2 per cent.

*Bichloride of mercury* is one of the most useful of salts employed in fixing. It has a strong shrinking action and is therefore very rarely used alone, but in combination with acetic acid enters into some of the most commonly used fixatives. The simplest of these is a saturated solution of mercuric chloride in water, to which is added 5 per cent glacial acetic acid. The relative proportions of sublimate and acetic acid should be determined in each case experimentally, adjusting the proportion of acetic acid so as to balance the shrinking action of the sublimate. Sometimes the sublimate is dissolved in alcohol instead of in water and in this way a greater concentration may be obtained. Great care is required

in washing out the sublimate from tissues after fixation. If this is not completely accomplished, crystals remain in the tissue which are often of such nature as to be mistaken for normal cell structures. The addition of iodine to the washing fluids, particularly the higher alcohols, facilitates the solution of the bichloride. When the iodine ceases to be decolorized, the sublimate has been completely removed.

*b. Advantages.* These substances offer a considerable range of effects and, by forming suitable combinations, aid materially in the differentiation of various cellular and tissue structures. In addition they also act as mordants for stains and improve the precision of differential staining.

*c. Disadvantages.* The disadvantages of these solids in solution are that they may produce deposits, as in the case of mercuric chloride particularly, and they also require a considerable degree of washing in order to remove the uncombined portion.

**3. Combinations of Agents in Solution.** *General Principles Involved in Choice of Combining Agents.* Such combinations are much more common as fixatives than are single substances. Formulas for these will be given under the main constituents. It may perhaps be well here, however, to note the general principles which should be followed in forming combinations. It is obviously undesirable to introduce complexity when a simple solution will suffice.

*Balancing, Shrinking, and Swelling Effects.* In general it may be said that a combination to be effective should balance the swelling action of one reagent by the shrinking effect of another, as when the shrinking action of mercuric chloride or picric acid is counteracted by the swelling action of acetic acid.

*Providing a Wide Range of Application by Varying the Proportions of Elements.* Because of these antagonistic effects, it is possible, by regulating the proportions of the reagents, to adapt the action of particular combinations to the peculiarities of various tissues. Where three elements are united, the range of application is in some degree further extended. It is only by experimenting with a particular material that an appropriate fixative can be developed. Although we are dependent upon this empirical method, it nevertheless provides a means by which almost any cell element or tissue can be accurately preserved. The range of any one combination is, of course, dependent upon the constituent elements, and some of these are capable of much wider application than others. Of all the combinations so far developed, those containing picric and acetic acids and formol appear to have the greatest flexibility.

*Acetic Acid Combinations.* Acetic acid is so universal in its use that it enters into nearly all mixtures. Formulas including it will therefore

be found under various headings indicating other active constituents with which it is combined.

*Bichloride of Mercury Combinations* (See also Zenker's Fluid).

(1) *Gilson's fluid*

Nitric acid, 46° strength, about an 80 per cent solution . . .	15 c.c.
Glacial acetic acid . . . . .	4 c.c.
60 per cent alcohol . . . . .	100 c.c.
Distilled water . . . . .	880 c.c.
Mercuric chloride . . . . .	20 gm.

This is a rather unusual combination but on some materials it produces excellent results. It should be given a trial when difficulties obtain in securing a good fixation with other mixtures. It fixes rapidly but does not commonly overfix, and in the case of amphibian eggs, if allowed to act for several days, will dissolve off the albumen.

(2) *Worcester's fluid*

10 per cent formalin saturated with mercuric chloride . . . .	9 parts
Glacial acetic acid . . . . .	1 part

This is an excellent fixative which deserves wider use.

(3) *Heidenhain's trichloracetic with sublimate*<sup>1</sup>

Saturated aqueous solution mercuric chloride . . . . .	100 parts
Trichloracetic acid . . . . .	2 parts
Glacial acetic acid . . . . .	1 part

(4) *Formol-sublimate*

10 per cent formol saturated with mercuric chloride

(5) *Sublimate acetic*

Saturated aqueous solution of mercuric chloride . . . . .	95 parts
Glacial acetic acid . . . . .	5 parts

Sublimate fixatives are rapid and vigorous in action and are best used on small objects for short periods of time. With most stains they give sharp and brilliant pictures. They require careful washing, which may be facilitated by the presence of iodine in the higher grades of alcohol.

*Bichromate Mixtures.* The original of these combinations is Mueller's fluid. This is prepared as follows:

(1) *Mueller's Fluid.*

Potassium bichromate . . . . .	25 gm.
Sodium sulphate . . . . .	10 gm.
Water . . . . .	1000 c.c.

This is now rarely used for fixing, but is an excellent hardening

<sup>1</sup> Heidenhain, M. *Ztschr. f. wiss. Mikroskop.*, 25: 405, 1909.

agent. There seems to be no reason for the addition of sulphate of soda and it might just as well be omitted.

(2) *Zenker's Fluid*. This is a modification of Mueller's fluid, produced by adding to it 5 per cent of mercuric chloride and 5 per cent of glacial acetic acid. Fix ten to twelve hours; wash with water. The sublimate may finally be removed by adding iodine to the washing fluid, preferably to the alcohol in which the sections are dehydrated. Too long a fixation produces deposits of crystals which are difficult to remove.

(3) *Helly's Fluid; or Zenker-formol*. This is a modification of Zenker in which the acetic acid is replaced by 5 per cent formalin. This fixative gives very beautiful results but in some materials extensive distortions are produced when paraffin sections are made. The same material cut in collodion gives very beautiful and precise figures. It is particularly recommended for blood work, both on tissues and smears.

(4) *Tellyesniczky's Fluid*.<sup>2</sup>

Bichromate of potash.....	3 gm.
Water .....	100 c.c.
Glacial acetic acid.....	5 c.c.
Fix twenty-four to forty-eight hours. Wash in water.	

*Alcohol Mixtures.*

(1) *Carnoy's fluid*

1. Glacial acetic acid.....	1 part
Absolute alcohol .....	3 parts
2. Glacial acetic acid.....	1 part
Absolute alcohol .....	6 parts
Chloroform .....	3 parts

(2) *Carnoy-Lebrun*

Absolute alcohol .....	1 vol.
Glacial acetic acid.....	1 vol.
Chloroform .....	1 vol.
Saturate with mercuric chloride.	

This group of fixing fluids is an extremely valuable one because of the very good penetrating power possessed. Such difficult objects as eggs of *Ascaris megalocephala* and of insects are best fixed with these reagents. For cytological studies of nuclear structures, with the picro-formol-acetic mixtures and chrom-osmium-acetic combinations, they constitute a resource of almost universal application. Fixation is rapid. Wash in 95 per cent alcohol.

*Chromic Acid Mixtures.* The chromo-acetic combination given by

<sup>2</sup> Tellyesniczky. *Arch. mikr. Anat.*, 34: 52, 1889.

Flemming consists of 0.25 per cent of chromic acid and 0.1 per cent of acetic acid in water. A stronger solution consists of

1 per cent aqueous solution of chromic acid.....	95 parts
Glacial acetic acid.....	5 parts

(See osmic acid mixtures for formulas in which chromic acid appears, p. 560.)

(1) *Destin's fluid*

1 per cent aqueous solution of chromic acid.....	99 c.c.
Formol .....	6 c.c.
Glacial acetic acid.....	2 c.c.

Allow the brown fluid to stand a few days until it becomes green before use.

*Nitric Acid Mixtures.* The most frequently used combination of nitric acid is that of Perenyi which consists of nitric acid in 10 per cent solution, 4 parts; alcohol 95 per cent, 3 parts; chromic acid, 0.5 per cent, 3 parts. This is in some cases a very valuable fixative but seems rather uncertain in its reaction, possibly because of chemical changes which it undergoes on standing.

(1) *Formol-nitric.* A combination which has proved very valuable, especially in the fixation of chick embryos, is the following:

Formol, 10 per cent.....	3 parts
Nitric acid, 10 per cent.....	1 part

This is best applied by opening the shell of the egg, removing the superficial albumen, and then adding the fixative directly to the embryo. After ten minutes the blastodisc may be cut out with scissors and removed to a watch glass where it is hardened with formol sublimate.

*Osmic Acid Mixtures.*

(1) *Flemming's fluid.* Strong formula

(a) Chromic acid, 1 per cent aqueous solution.....	11 parts
Glacial acetic acid.....	1 part
Distilled water .....	4 parts
(b) Osmic acid, 2 per cent in 1 per cent chromic acid solution.	

Just before using mix 4 parts of (a) with one part of (b). Use 10 times the volume of the object. Fix two to seventy-two hours. Wash in water twenty-four hours.

A precise and delicate fixative for cytological details and adapted to many kinds of protoplasm. It may in some cases be used to advantage at low temperatures—0°C. for instance. Does not penetrate deeply and sometimes overfixes on the periphery of the mass and underfixes in the center. May be used advantageously when staining in iron hematoxylin or with aniline dyes (see also p. 604).



(2) *Lillie's chrom-osmic-acetic mixture*

Chromic acid, $\frac{1}{2}$ per cent solution.....	15 c.c.
Osmic acid, 2 per cent aqueous solution.....	$3\frac{1}{2}$ c.c.
Glacial acetic acid.....	3 drops

This is recommended by Lillie for fixing echinoderm eggs in order to follow the exact changes during fertilization.

(3) *Bichromate-chromic-osmic acid mixture of Champy.*<sup>3</sup>

3 per cent aqueous solution of bichromate of potash.....	7 parts
1 per cent chromic acid aqueous solution.....	7 parts
Osmic acid, 2 per cent aqueous solution.....	4 parts

Fix six to twenty-four hours. Wash in water for same period of time. Stain with iron hematoxylin.

(4) *Platino-aceto-osmic acid mixture of Hermann.*<sup>4</sup>

Platinic chloride, 1 per cent aqueous solution.....	15 parts
Glacial acetic acid.....	1 part
Osmic acid, 2 per cent aqueous solution.....	2 parts

Fix twelve to twenty-four hours. Wash for similar period of time in water. This in effect is very similar to Flemming's fluid. For a nuclear stain follow with iron hematoxylin. Chromosomes appear larger upon fixation in Hermann's fluid than in Flemming's. It is more difficult to secure a good cytoplasmic stain than after Flemming.

*Platinic Chloride Mixtures.*(1) *Juel's fluid*

Chromic acid, 2 per cent aqueous.....	25 c.c.
Platinic chloride, 10 per cent.....	2.5 gm.
Glacial acetic acid.....	1 c.c.
Water.....	75 c.c.

(2) *Merkel's fluid* (F. E. V. Smith's modification)

Acetic acid, 5 per cent.....	100 c.c.
Platinic chloride, 1 per cent.....	5 c.c.
Chromic acid, 1 per cent.....	10 c.c.

*Picro-formol-acetic Mixtures Used at the University of Pennsylvania.*(1) *Bouin's fluid*

The original picro-formol-acetic combination is that of Bouin. It is prepared as follows:

Saturated aqueous solution of picric acid.....	75 parts
Formol, C.P. ....	25 parts
Acetic acid, glacial.....	5 parts

(2) *Modifications of Bouin.* A series of experiments was carried out at the University of Pennsylvania, using a large number of combinations

<sup>3</sup> Champy, C. *Arch. de zool. expér. et gén.*, 52: 13, 1913.

<sup>4</sup> Hermann, F. *Arch. f. mikr. Anat.*, 34: 81, 1889.

of the three elements contained in Bouin's fluid, to which other substances were added. These were employed particularly for the finest cytological details and it was found that some improvements could be effected by modifications of the original formula. Those of most value have been described in the literature as P.F.A.<sub>3</sub> and P.F.A.<sub>15</sub> and, when combined with chromic acid, are marked with the designation B-3 and B-15. Preparation is as follows:

(a) *Allen's fluid* P.F.A.<sub>3</sub>

Picric acid, saturated aqueous solution.....	75 parts
Formalin, C.P. ....	15 parts
Glacial acetic acid.....	10 parts
Urea .....	1 part

(b) B<sub>3</sub> consists of this fluid to which is added chromic acid, 1 part.

(c) P.F.A.<sub>15</sub> (B 15) is the original Bouin's fluid to which are added two parts of urea and 1.5 parts of chromic acid.

The fluids with chromic acid turn green in perhaps half an hour, and after this change takes place they do not seem so efficient. The purity of the chemicals is extremely important. If a precipitate forms upon the addition of the urea, either it has not been stirred in carefully, or the formol is impure. If the color is blackish instead of a deep reddish brown the trouble is probably with either the formol or the chromic acid.

The addition of chromic acid to the mixture requires that it be used immediately, since rapid reduction of the chromic acid is effected by the formol. It is best, therefore, to add the chromic acid only at the time that fixation takes place. The P.F.A. mixtures without chromic acid are very valuable since material may be left in them for long periods of time without danger of overfixation. This is particularly true of the combination P.F.A.<sub>3</sub> and it has been found in the case of Orthopteran material that whole animals, which have been opened sufficiently for it to enter readily, may be so treated that the intimate cytological details of the cells, the germ cells particularly, are well preserved. This is a very great convenience when rapid preparation in the field is necessary. (For further discussion of modified P.F.A. mixtures see p. 249.)

	Bouin	B-15	3	B-3	2	8	16
Picric acid sat. aq. sol. ....	75	75	75	75	75	90	50
Formol.....	25	25	15	15	10	5	20
Acetic acid, glacial.....	5	5	10	10	10	5	5
Urea.....	..	2	1	1	..	..	1
Chromic acid.....	..	1.5	..	1	..	..	1
Water.....	..	..	..	..	..	..	25

(3) *Picro-sulphuric acid*<sup>a</sup>

Distilled water .....	100 vol.
Sulphuric acid .....	2 vol.
Saturate this mixture with picric acid.	

This was formerly much used in embryological work, but it has been found less desirable than the picro-formol-acetic mixtures.

(4) *Picro-acetic*<sup>a</sup>

Saturated aqueous solution of picric acid .....	100 parts
Water .....	200 parts
Glacial acetic acid .....	1 part

4. **Vapors.** For very delicate objects, present in a small quantity of water or disposed as a thin film, fixation may be secured by exposure to the vapors of certain substances. Principally available here are formaldehyde and osmium tetroxide. To secure action in this manner it is sufficient to bring the object into a small chamber containing the gaseous reagent. Films on cover glasses are conveniently inverted over a vessel containing a solution from which the gas arises. In the case of osmium tetroxide a few drops of Flemming's fluid in a small Stender dish will provide a ready means of fixing smears on covers. When it is desired to fix objects under observation this may be done by observing them on an inverted cover over a moist chamber into which the vapor may be drawn when desired. Fixation by vapors is a precise, delicate and ready method when it is applicable.

## B. DIFFERENTIAL EFFECTS OF FIXATIVES

These may be considered under several headings:

## I. Selective Action

There is the question of their selective action. There seems to be no good reason for classifying fixatives as cytological, histological, or embryological in application because, with good powers of penetration, well-balanced combinations such as the picro-formol-acetic mixtures, will preserve cells, tissues, or organs almost equally well. However, there do exist certain selective actions between parts of cells and particular reagents, so that for certain cytoplasmic structures ordinary nuclear fixatives are regarded as unsatisfactory. Such differences nevertheless may, in part at least, be the result of method of after-treatment. It is com-

<sup>a</sup> Kleinenberg. *J. Mikr. Sci.*, 1879.

<sup>a</sup> Boveri. *Sitzungsb. d. Gesellsch. f. Morphol. u. Physiol.*, 2: 101, 1886.

monly stated that the presence of acetic acid in any combination is inimical to the preservation of mitochondrial structures. It has been found by experiments on Orthopteran spermatocytes that Flemming's fluid, if only lightly washed out, is a fixative which, followed by hematoxylin, presents an almost perfect mitochondrial stain. The elements have not been removed by the acetic acid, as supposed, but commonly after long-continued washing when a good nuclear stain results, the mordanting action of the fixative has been suppressed and the mitochondria do not retain the hematoxylin. The experiment by which this demonstration was carried out is as follows:

A Flemming-fixed preparation of grasshopper testis, after the sections were spread and dried, had the paraffin removed from one end of the slide by xylol. The whole preparation was then placed in 70 per cent alcohol for several days, after which the paraffin was removed from the remaining sections and the whole preparation stained with iron hematoxylin. On examination, the washed-out sections were found to have clear, stained nuclei with no mitochondria showing, while those which had been protected by the paraffin and were only slightly washed showed a reverse staining reaction with the mitochondria dark and the chromatin unstained. It is thus apparent that the selective action of any fixative is in a measure dependent upon the after-treatment of the material.

Taking all of these facts into consideration, therefore, as a practical matter, the differentiation of fixatives into nuclear and cytosomic is justified. We may well question, however, whether the effects produced upon the cytoplasmic elements are due to the absence of acetic acid or to the mordanting action of other substances in the mixture. Most of the methods adapted to mitochondrial or Golgi apparatus constituents require extended action of the fixatives, even at high temperatures, both of which conditions facilitate mordanting action. In general, unless stated otherwise, the fixatives given are adapted to observation of nuclear structures, and those employed for cytoplasmic constituents are separately considered (see also *Cytological Methods*, p. 265).

## II. Penetration

Further differential effects may be due to the rate and degree of penetration of the agents in the fixative.

**1, 2. Rate and Degree.** These are particularly marked in the case of Flemming's fluid. The acetic acid enters rapidly and well, while the osmic acid is restricted in its action to the superficial layers of cells. Because of this difference in character of the penetration, a piece of tissue

presents very often a peripheral zone in which the protoplasm is glassy and homogeneous while the central portion stains with characteristic differentiation between the cell elements. In any combination, therefore, it is necessary to know the physical characteristics of the elements which enter into it if the best results are to be obtained from its use.

### III. Influence of Physical Condition

The differential effects are also due to the physical condition of both the specimen and the medium.

1. **In Specimens.** *Size and Density.* Conditions of the specimen which influence action of the fixative are size and density. In choosing a fixative, therefore, the nature of the tissue must be carefully studied. Very dense or resistant substances require reagents of vigorous action and rapid penetration, whereas loose tissues may be fixed with comparatively mild ones.

2. **In Medium.** The physical state of the medium in regard to temperature, concentration and the presence of adjuvants, has much to do with the differential effect of the fixative. It has long been recognized that elevation of temperature facilitates rapid penetration, but it has not been so well recognized that this produces a difference in its action on various cell elements. In a series of experiments conducted at the University of Pennsylvania it was discovered that the relative density of nucleus and cytoplasm may be varied by the temperature of the fixative.

a. *Temperature.* In extreme cases the nucleus appears practically empty, because of the solvent action of the fixative, but by the application of Flemming's fluid at very low temperatures, in the presence of urea, the nucleus of grasshopper spermatocytes is considerably denser than the cytoplasm. Apparently a better cytological fixation is secured by the use of Flemming's fluid at 0°C., while picro-formol-acetic combinations do better at a temperature of 35 to 40°C.

b. *Concentration.* The concentration of the fixative, as a whole, as well as the proportion of its parts, also influences its selective action. Only very recently, however, has it been realized that the presence of relatively inert substances, such as urea, various sugars, etc., has a pronounced influence upon the operation of the fixative. The action of urea has already been referred to, it being the first of such substances employed. Some experiments upon plant cells with different sugars indicate that they have specific effects. For instance, glucose added to Flemming's fluid produces in each cell of the *Podophyllum* root tip a large clear vacuole, whereas if levulose is substituted for the glucose no such vacuole is seen.

It thus appeared that these sugars, having such specific actions, might be advantageously employed in the fixing of plant tissues. An extension of these experiments was therefore carried out in the Botanical Laboratory, University of Pennsylvania, and will be found discussed more fully in the chapter on Botanical Methods (p. 207).

*c. Adjuvants.* So far, the studies upon differential action of adjuvants in fixatives have been of a preliminary sort, but they indicate strongly the value of such substances and the need for a more complete investigation of their action. Some early studies by McClendon<sup>7</sup> showed that the swelling and cytolysis of cells in the convoluted tubules of the kidney could be prevented by adding 10 to 40 per cent of sugar to the fixative. The small percentage of urea and sugar employed in our experiments would not seem to operate by affecting the density of the reagent, as in McClendon's experiments, and we can only suppose that some more exact adaptation is involved in their specific effects. Since these are substances which penetrate protoplasm with unusual rapidity it may be surmised that their action is, in part at least, due to this property.

### C. METHODS OF APPLICATION

The methods of application of fixatives may be considered in relation to the kind of material. Small pieces of tissue may be immersed directly in the fixing fluid, whereas if large organs or whole animals are to be fixed, the reagent should be injected through the circulatory system.

#### I. By Immersion

If fixation is by immersion, regard must be had for the following circumstances:

**1. Proportionate Volumes.** The volume of the fixing fluid in relation to that of the tissue must be such that the water abstracted from the tissues will not so dilute the fixative as injuriously to affect its action. This proportion varies with the particular fixative employed. In general there should be a volume of the fixing fluid 25 to 50 times that of the object. It is also desirable sometimes to substitute fresh fixative, especially if it is applied for considerable periods of time.

**2. Position of Specimen in Fluid.** The position of the specimen in the fluid is not unimportant. If it is possible to suspend the object near the surface of the medium it is advantageous, because in this position it is always in a reagent of high concentration. If the specimen has been attached to a small piece of paper it will float on the surface un-

<sup>7</sup> McClendon, J. F. *Anal. Record*, 7: 51, 1913.

aided. Larger objects may be suspended by a thread in a cylindrical vessel.

**3. Duration of Action.** The duration of action required varies with the material and the particular fixative. There are some reagents which produce injurious effects by overfixation, whereas others may be allowed to act indefinitely. The minimum time for effective results can be determined only by experiment, but as a rough guide it may be said that a piece of tissue not over 5 mm. in diameter in chromic acid combinations will be fixed within a period of two to twelve hours. The picro-formol-acetic combinations (without chromic acid) may be allowed to act for almost any length of time without injury to the specimen. Rapid and vigorous fixatives, such as those of Carnoy, fix within an hour in some cases.

## II. Fixation by Injection

If the presence of blood is not essential, a better picture of the relationships of all other tissues to each other, as well as of chromosomes, may be obtained in complex organs, as brain, testis, etc., by injecting the fixing fluid rather than by cutting the organ into small pieces. In the case of testes which have a small quantity of interstitial tissue, as the rat's, this tissue is not torn.<sup>8</sup> Any fixative may be injected.

The blood must first be removed by washing out the vessels with Locke's or normal salt solution, which for mammals is 0.9 per cent. The washing is begun while the animal's heart is still beating, if possible; consequently light etherization is essential. As soon as the blood is out of the veins the fixing fluid is injected. Each fluid may be forced in by gravity or by air pressure. Too great pressure is to be avoided throughout the process, as extravasation is easily produced. A pressure equal to about 10 mm. of mercury is sufficient for a white rat. It may be obtained through gravity by elevating the container of the fluids to a height of about 25 to 30 inches. A funnel, supported on a retort stand, answers the purpose fairly well. This is connected to the cannula by rubber tubing. A screw clamp will control the rate of flow. Some workers advocate a pulsating flow, imitating the heart beat, but the writer has found a steady flow perfectly satisfactory.

A more elaborate apparatus, by which the pressure may be varied, is shown in Figure 1 (p. 254). This consists of a pressure bottle A, and an aspirator bottle B for holding the fluids. The delivery tube D.T. from B carries the fluid through the warm water in C to the cannula. The flow is controlled by the screw clamp D. In order to free the fluid from any air

<sup>8</sup> Allen, E. *Anat. Record*, 16: 25, 1919.

bubbles which might stop the flow of the fluid in the blood vessels, a vent is introduced by the T-tube, controlled by clamp *b*. The atomizer bulb is used to force air into A. The resulting pressure is communicated to the fluid in B through the connecting tube *c.t.*, and controlled by the screw clamp *a*. The mercury manometer registers the pressure.

Instead of the atomizer bulb, one may use a bottle of water elevated about 2 feet and connected by tubing to A, as shown in Figure 2 (p. 255). The Woulff bottle may be replaced by a salt mouth bottle fitted with a three-hole rubber stopper. Or, if air pressure is supplied in the laboratory, it may be used under proper control. In this case, a manometer is absolutely essential. If desired, 2 B bottles may be used. A Y-tube will connect both to the delivery tube; flow from each is controlled by a screw clamp. For use, disconnect the tube *c.t.* from the bottle containing the salt solution and connect with the other. Use rubber stoppers and heavy walled tubing throughout except for the short cannula tube, where flexibility is desirable.

**1. Steps in Injecting.** *a. Preparation of Apparatus.* Whatever form is decided upon, set it up fully and test it thoroughly before preparing the animal. Have several cannulas varying somewhat in size. If possible, try the cannulas on an experimental animal to be sure the right size is at hand. Have ready means of keeping the animal at body temperature. For this purpose use a vessel of warm water under a dissecting pan, or a waterproof electrically heated pad or table. Have salt or Locke's solution and the fixing fluid at the right temperature, and be sure they can be held at that temperature. As there always is some loss of heat in the tubing, 40° to 45°C. is about right. When entering a mammal each fluid should be at about 37° or 38°C. As a source of heat a paraffin oven or a water bath is much better than a gas burner, as the latter must be watched lest it overheat the fluid. The fixing solution should be well stoppered while being heated and while in use. Have something to place under the animal's shoulders, as working in the chest cavity is much easier if it is elevated. The head may droop. Have dissecting tools ready and in place.

*b. Washing.* Having everything ready and the fluids at the proper temperature, let the salt solution flow down into the cannula while the animal is being etherized. Get rid of all air bubbles by letting them escape through the T-tube. Let a very slight flow continue, enough so that the cannula will be full when held in any position. This flow will not interfere with its insertion into the blood vessel, and guards against clotting.

Lay the animal on its back, the heart still beating, and make a longitudinal midventral incision through the skin from the genital outlet



to the neck. The hind legs may be tied fast and the front legs tied behind the back. Slit the abdominal muscles to the breastbone, and cut the ribs a little to the left of the breastbone with care not to cut the mammary artery. Bend the ribs back, breaking them if necessary, and even removing the ventral ends, to have a clear cavity in which to work. The slight loss of blood is of no consequence. Cut the left side of the diaphragm from the ribs. The heart should still be beating. If the whole animal or only the anterior end is to be used, inject through the heart. If only the hinder part is wanted insert the cannula in the thoracic aorta.

Suppose the testes of a rat are to be fixed with warm B-15. After securing the cannula firmly in the thoracic aorta, start the flow of salt solution with a very gentle pressure, merely sufficient to distend the artery posterior to the cannula. Stop the pressure by closing the valve *b*, and open the posterior vena cava to let the blood escape. It may be absorbed in the cavity by absorbent cotton, but keep it loose to avoid back pressure. Now turn on the pressure gradually but quickly to 20 or 25 mm. mercury counting the movement in both arms of the manometer. The testes should be in the scrotum. If retracted, push them back. They do not seem to inject well if in the body cavity. Slit open the scrotum on one side to observe the loss of blood in the prominent testicular vessel. Watch the intestines and the liver. By the time this last-named organ is pale the blood is about washed out. If these organs do not pale, the flow is interrupted. If one testis is pale, the other is almost certain to be washing satisfactorily if it is in its natural position in the scrotum.

*c. Injecting the Fixative.* To change to the fixing fluid, close the clamps *d* and *a*. If two aspirator bottles have been provided, close the clamp for the salt solution, change the connecting tube to the other bottle, open its discharge clamp and clamps *a* and *b*. Start the pressure gently, and continue until the salt solution in the delivery tube has all been replaced by the fixing solution, and all air bubbles have escaped; then close clamp *b* and open *d*. Increase the pressure as before. In this case the fixative is colored, and the exposed testis, the intestines and the liver should quickly appear yellow, and the fixative should escape from the vena cava. Continue the flow until about 100 c.c. have passed through. Let more through if the flow still continues. Toward the latter part of the process the pressure may be increased slightly without injury to the tissues. The hinder parts of the animal will be stiffened by the formol. To insure preliminary fixation in a rat 100 c.c. is sufficient.

*d. After-treatment before Dehydration.* Remove the testes, still

covered with the tunica albuginea, and also the epididymis if desired, and place in fresh fixative at 38°C. After about thirty minutes slice the testes with a thin safety razor blade into 3 or more pieces, preferably longitudinally, and return the tissue to the fixative for another half hour. Longer fixation in this fluid is undesirable for these organs. They may be placed in Bouin if for any reason the dehydration process is to be delayed.

*e. Injecting through the Heart.* In this case a larger cannula is used. Inject through the left ventricle for either the anterior end or the whole animal. If the lungs are not desired, let the blood escape from the right auricle; if they also are to be injected, clamp the opening in the right auricle, removing the cannula to the right auricle. Two animals may be needed for both purposes if they are small.

If a colorless fixing fluid is to be used, a little eosin will serve to mark its progress, unless the fixative decolorizes the eosin rapidly.

#### D. RELATION TO AFTER-PROCESSES

##### I. Washing

1. **Medium.** The selection of the medium for washing is determined by the nature of the fixative employed. It is always emphasized that after picric acid combinations the tissue should not be allowed to remain long in water because of the macerating effect which it exercises, whereas the chromic acid combinations are so vigorous in action that water will not injure the specimens during a period of even twenty-four hours. As a general rule it may be noted that the sooner the material is brought into 70 per cent alcohol the better. Most of the reagents are soluble in alcohol, so that after a preliminary washing the specimen can be run up into that grade and by changing this repeatedly the excess fixative may be removed. Certain combinations not aqueous in their nature, such as Carnoy's mixtures, should be run into an appropriate grade of alcohol at once without passing through water. The rule is to proceed from the fixative into a concentration of alcohol somewhat similar to that of the fixative.

2. **Method of Application.** It is sometimes sufficient to place the fixed material directly in the washing medium in any convenient vessel, but where small objects are handled, especially in large numbers, it is often helpful to utilize special pieces of apparatus. A number of these have been devised and consist either of small vessels having perforated bottoms or cylinders capped with porous fabric. By causing a current of

water to flow through, the specimens are washed rapidly without danger of loss. The following is a description of such an arrangement by Allen<sup>9</sup>:

*Automatic Device for Changing Fluids on Many Small Objects*

The apparatus (Fig. 1) is essentially a glass tube about 300 mm. long by 16 mm. inside diameter, supported at an angle of about 30°, fitted with a glass stop-cock at the lower end and with a 2 hole rubber stopper at the upper, through which fluids are conducted from the supply bottle. The overflow control tube fitted into the upper stopper serves two purposes: when the large glass tube is being filled from the supply bottle, it allows air to escape; it prevents overflow when the tube is full and the stop-cock is closed. A retort stand fitted with rings and clamps is adequate support. The supply bottle is of the aspirator type. The wads of cotton (cotton and c) insure the thorough mixing of the new fluid with that in the tube. The upper wad (c) should be on top of the tissue; the space between it and the intake gives opportunity for mixing before the new fluid reaches the tissue. If tissues are inclosed in small glass tubes, each with its number, and covered with wide-meshed cloth, it is well to place the tubes in lengthwise, and not to allow them to be tightly packed.

Tissues may be fixed, stained, washed, dehydrated, and cleared without removing them from the tube. The process may be interrupted at any step, and the tissues left in the appropriate fluid overnight or longer without loss by evaporation. Simply close the spigot. The rate of change is controlled by the rate of dropping. Used fluids may be preserved, account being kept on the labels of the number of times used. One may introduce 50 per cent alcohol into water; 95 per cent into 50 per cent; but it is better to use intermediate mixtures if cytological details are especially desired.

**3. Duration and Extent of Action.** The washing-out processes must be determined by the effect desired. Commonly it is best to remove all of the uncombined material, but sometimes for mordanting purposes, as indicated in the discussion of mitochondrial stains, the washing should not be complete. In general, if complete extraction of the uncombined fixative is desired, the length of the period should approximate that of the action of the fixative. If the medium employed is water, however, this should not extend beyond twenty-four hours as a rule.

## II. Staining

**1. Mordant Action.** As has been indicated in the discussion of the process of washing, fixation bears a definite relation to the after-process

<sup>9</sup> Allen, E. *Science*, 66: 427, 1927.

of staining. After most fixatives almost any nuclear stain may be applied, although there are differences in the degree of precision of the stain according to the nature of the fixative. The mercuric bichloride com-

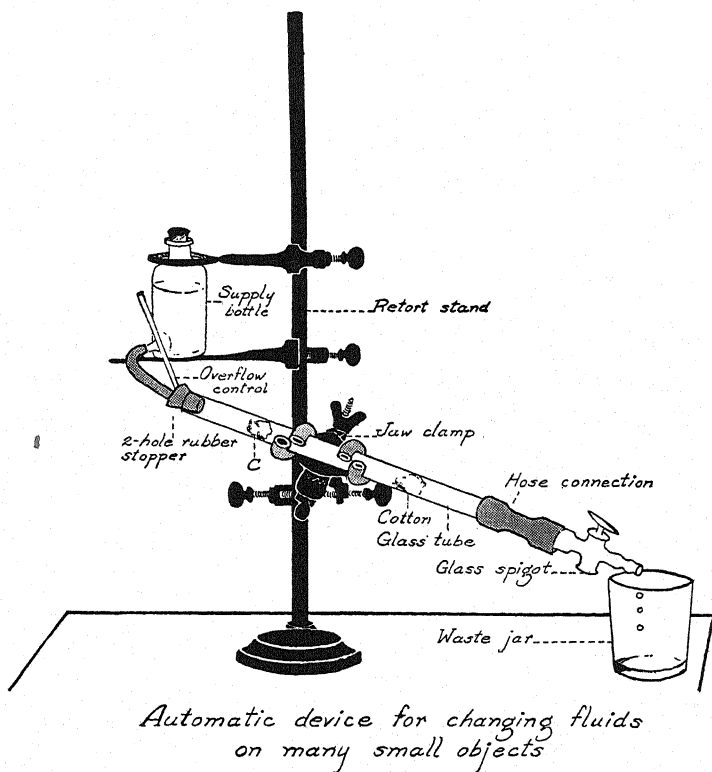


FIG. 1.

binations show a very brilliant reaction with hematoxylin stains. Certain of the aniline dyes do not operate well unless osmic or chromic acid has been present in the fixative. A further discussion of this subject will be found under the heading "Stains" (p. 604).

**2. Revival of Staining Capacity.** The condition in which cells are left by the fixing fluid is not stable. From the point of view of staining possibilities a slow deterioration sets in immediately, and the thinner the tissue the sooner a loss of staining power is noticeable. Thus, a block of tissue may remain in good condition for years, while a smear or connective tissue spread is less satisfactory in a few days. If the fixation was good in the first place and the deterioration is not too pronounced, staining capacity can be revived by the use of peroxides. Smears, mem-

branes, or sections not more than  $10\mu$  in thickness should be placed in commercial hydrogen peroxide for thirty minutes or more and then washed in water before being stained. A 10 per cent solution of benzoyl peroxide<sup>10</sup> (St. Szecsi, p. 329) in acetone is even more satisfactory and requires only fifteen minutes. It should be washed out in xylene-acetone (2 to 3), followed by alcohol. In the case of a faded slide which is worth restaining, it is advisable to soak off the coverglass and treat the preparation with benzoyl peroxide solution before putting it into the stain (Slider).

<sup>10</sup> Benzoyl peroxide is unstable if heated.

## CHAPTER X

### STAINS AND STAINING

C. E. McCLUNG AND H. J. CONN

NATURE OF STAINING AGENT 573. Origin or source 573. Aniline dyes 576. Composition of staining agents 605. NATURE OF STAINING COMBINATION 605. Physical nature 605. Chemical nature 606. APPLICATION OF STAINS 607. *Intra vitam* 607. *Post mortem* 607. FORMULAS 610. Aniline dyes 610. Carmine and Cochineal 611. Hematoxylin and hematein 613.

#### A. NATURE OF STAINING AGENT

##### I. Origin and Source

**I. Organic.** *a. Natural.* Natural organic dyes are (1) from an animal source (carmine) and (2) from plants (hematoxylin and brazilin).

*Carmine and Cochineal.* Carmine is the only animal product used in staining. Certain scale insects, particularly *Coccus cacti coccinillifera*, secrete a colored substance, cochineal, which is used commercially as well as scientifically. The dried bodies of these insects are ground up into a powder and in this condition may be dissolved in water to which is added either an acid or an alkali, thus forming directly a staining solution. Usually a plain aqueous solution is not employed, but has added to it certain adjuvants as in the case of hematoxylin.

While stains may thus be made directly from the crude material, in general the derived substance, carmine, is the basis of the stain. Carmine, however, is not a simple chemical compound but a complicated mixture of which the essential coloring agent is carminic acid. Therefore instead of using either the crude cochineal or the product obtained from it, carmine, the active staining principle, carminic acid, may form the base of the stain.

Carmine is a bright red substance. Cochineal inclines more to an orange red. Of all stains, these are most applicable for staining *in toto* because they do not overstain. Particularly in embryological work, the cochineal stains, applied before sectioning, are of very great value. Carmine stains in general are used progressively. As solvent media both water and alcohol are employed. Also they may be either acid or alkaline in reaction. Carmine is primarily a nuclear stain, but properly applied may be made to color selectively various parts of the cell.

*Brazilin* ( $C_{16}H_{14}O_5 + 11\frac{1}{2}H_2O$ ) is a natural dye obtained from Brazil wood, *Cesalpinia crista*. It is similar in general character to hematoxylin, but has a red color. It is not so active and strong a dye as hematoxylin and has had only a limited use. It is possible that more careful investigation of this dye might disclose more specific application than it has received. It may be handled in the same manner as hematoxylin, as either a progressive or a regressive stain. Like hematoxylin, brazilin acts in staining through the presence of an oxidized form—brasilin,  $C_6H_{12}O_5$ .

*Hematoxylin* ( $C_{16}H_{14}O_6$ ), is derived from logwood, *Campechianum*. It is best obtained by extracting the wood with water in the presence of ether. Usually, however, it is prepared by taking the logwood extract which is found in commerce and treating it with ether. The process is a rather long and difficult one and for some reason the products of successive operations differ in their physical characteristics. Whether there is any chemical variation is not known. As found on the market hematoxylin is in the form of small crystals, sometimes acicular. Some samples formerly on the market were very white, a result produced by using sulphur dioxide in the process of manufacture. This practice is undesirable, since it injuriously affects the keeping qualities of solutions, and has been abandoned. Commonly crystals range in color from light yellow to a rusty purple. In the dry condition the material keeps indefinitely. If combined with one molecule of water the crystal form is rhombic, with three molecules it is tetragonal.

Hematoxylin is soluble in water, glycerin and alcohol. It is commonly used in aqueous solutions either plain or combined with a mordant. Aqueous solutions are most readily prepared by first dissolving the hematoxylin crystals in a small quantity of 95 per cent alcohol and then adding water in sufficient quantity to make the solution of the right concentration, usually  $\frac{1}{2}$  per cent. Such solutions do not keep indefinitely and during the development of American hematoxylin much difficulty has been encountered in preparing it so that it would remain usable for any length of time. It has been found that the addition of a very small quantity of a sulphite, such as sodium bisulphite, will preserve the solution in good condition very much longer. This prevents oxidation, of course, which is not a drawback in Heidenhain's technique. In case such a solution is employed in a progressive stain the preservative action of the sulphite must be overcome by some oxidizing substance.

Hematoxylin does not stain directly, but only after it has been oxidized to hematein, and in stains operating progressively this process must occur before use. In most cases this is accomplished by allowing it to "ripen," which is a process of slow oxidation. It may be more readily and accurately accomplished by the addition of a small quantity of hy-

drogen peroxide to the solution. The color depends upon the degree of oxidation, hematein,  $C_{16}H_{12}O_6H_2O$ , producing a blue color while dioxyhematein, trioxyhematein and tetraoxyhematein are progressively browner. Apparently the best form for use is the trioxyhematein. The higher compounds are less precise in action and less agreeable in color. A hematein is now available commercially which seems entirely satisfactory.

In regressive stains, such as Heidenhain's iron-hematoxylin, the tissue is first mordanted. Subsequently, when immersing it in a hematoxylin solution, a lake is formed between the mordant and the staining agent and this becomes fixed in the elements of the tissue.

Hematoxylin is not a good agent for in toto staining, but it is probably the very best of our dyes for sections. It may be applied either progressively as in the case of Delafield's hematoxylin, or regressively in the classical iron alum-hematoxylin method of Heidenhain.

The color effects in tissues produced by the application of hematoxylin vary with the character of the medium in which it is dissolved. In the presence of acids the color is red. In the presence of alkalies it is blue. Commonly, because tap water in which sections are washed is slightly alkaline, the final result is blue. Fresh solutions, with their lower oxidation products of hematein, give bluer effects than the same ones after use. Finally, in old baths, only a rusty, greenish black color results. In neutral balsam, unchanged by age, the color of hematoxylin in sections is permanent. Of all the stains available to the microscopist, hematoxylin is the most generally useful because of its power, selectivity, precision and permanence, in addition to its pleasing color. Because of its particular affinity for chromatin with ordinary fixations and for cytoplasmic constituents under modified techniques, it is invaluable in cytological studies. It is also very adaptable to different mordants and may be combined with various compounds containing aluminum, iron, chromium, molybdenum, etc., to form stains for specific purposes.

*b. Synthetic Dyes.* Aniline dyes. These are classified, according to their chemical behavior, as basic and acid. Basic anilines are the ones having the staining radical in the position of the base in a salt and they therefore operate upon the acid parts of the cell like the chromatin and nucleus. Acid dyes, on the contrary, are those in which the radical is in the position of the acid in the salt. There are a great many aniline dyes, but for most purposes a limited number are entirely satisfactory. The basic aniline colors most in use are Bismarck brown, fuchsin (basic), gentian violet or crystal violet, Janus green B, methyl green, methylene blue, neutral red, safranin, thionin, toluidine blue. The acid dyes most in use are Bordeaux red, Congo red, eosin, fuchsin (acid), orange G,



Sudan III, trypan blue, aniline blue, water soluble light green, fast green. A full discussion of the characteristics of aniline dyes by Conn follows.

### Aniline Dyes<sup>1</sup>

Outstanding information concerning the nature and uses of biological stains has been gathered together here. Very little information is given concerning the chemistry of the dyes, and nothing concerning the technique by which they are employed. For the former information one should consult other authorities; for the latter, other sections of this book.

The dyes are listed below in alphabetical order, as this seems the arrangement by means of which the biologist can most readily find any dye he may desire. The confusing synonymy of dyes often has made it necessary to list the same dye in several places; but the description of the dye is given only under the one name which has been adopted by the Commission on Standardization of Stains as the preferred designation.

For further detail along the same line as given below the worker is referred to the book "Biological Stains."<sup>2</sup> For technique the other sections of this book may be consulted and also such standard texts as "Krause's Enzyklopädie,"<sup>3</sup> Lee's "Microtomet's Vade-Mecum,"<sup>4</sup> and Mallory and Wright's "Pathological Technic."<sup>5</sup>

**Acid Bordeaux.** See Bordeaux red.

**Acid Congo R.** See vital red.

**Acid Fuchsin.** See fuchsin, acid.

**Acid Green.** See light green SF, yellowish.

**Acid Green O.** See naphthol green B.

**Acid Magenta.** See fuchsin, acid.

**Acid Orange.** See orange II.

**Acid Phloxine GR.** See chromotrope 2R.

**Acid Rubin.** See fuchsin, acid.

**Acid Yellow R.** See metanil yellow.

**Acridlavine.** A basic dye of the acridine series, chiefly employed as an antiseptic. Also added to bacteriological media for its bacteriostatic action.

**Alcohol Soluble Eosin.** See ethyl eosin.

**Alizarin.** An acid dye of the oxyquinone group, formerly extracted

<sup>1</sup> Section on Aniline Dyes by H. J. Conn.

<sup>2</sup> Conn, H. J. *Biological Stains*. Geneva, N. Y., Ed. 3, 1936.

<sup>3</sup> Krause, *Enzyklopädie der Mikroskopischen Technik*. Ed. 3. Berl., 1926, 1927.

<sup>4</sup> Lee, A. B. *The Microtomet's Vade-Mecum*. Ed. 9. London, 1928.

<sup>5</sup> Mallory, F. B. and Wright, J. H. *Pathological Technic*. Ed. 8. Phila., 1924.

from madder root, but now prepared synthetically. It stains tissues a feeble yellowish red if used on them directly. In the presence of aluminum compounds, intense red colors are formed; bluish violet in the presence of iron; and brownish violet in the presence of chromium. It has been used as stain for nervous tissue, but its chief present use is as an indicator.

**Alizarin Blue RBW.** See gallocyanin.

**Alizarin Carmine.** See alizarin red S.

**Alizarin No. 6.** See purpurin.

**Alizarin Purpurin.** See purpurin.

**Alizarin Red, Water Sol.** See alizarin red S.

**Alizarin Red S.** (Syn. *Alizarin red, water sol. Alizarin carmine. Alizarin sulphate.*) A strongly acid dye, sodium alizarin sulphonate. It has been used by Benda for staining chromatin in preparations in which the mitochondria are stained with crystal violet. It has also been used as a vital stain for nervous tissue in small invertebrates, and proves to be a good stain for bone.

**Alizarin Sulphate.** See alizarin red S.

**Amanil Garnet H.** See Erie garnet B.

**Amaranth.** (Syn. *Naphthol red. Fast red. Bordeaux. Bordeaux SF. Victoria rubin. Azo rubin. Wool red.*) A strongly acid red dye of the azo series, having little value as a protoplasm stain. It has been employed for staining axis cylinders, but is not one of the commonly used stains.

**Amethyst Violet.** (Syn. *Heliotrope B. Iris violet.*) A basic dye, tetra-ethyl pheno-safranin. It is seldom employed for biological staining, but has been recommended by Ehrlich and Lazarus in certain triple staining procedures.

**Aniline Blue, Alc. Sol.** See spirit blue.

**Aniline Blue, Water Sol.** (Syn. *China blue. Soluble blue 3M or 2R. Marine blue. Cotton blue. Water blue. Berlin blue.*) A strongly acid dye of the triphenyl methane series, derived from spirit blue by sulphonation; it is always a mixture, one of the components of which is methyl blue (q. v.). Unfortunately it is a dye whose exact composition cannot be controlled by the present methods of manufacture; it is therefore impossible to be sure of the identity of any two batches, even though made by the same manufacturer. For this reason it is a rather unsatisfactory dye to employ in any delicate staining procedure.

It is a widely used histological stain, having valuable properties as a counterstain. It is also employed as an indicator, but is much less satisfactory for the purpose than the sulphonephthaleins. The best known procedure at present for which it is required is the Mallory connective tissue stain, in which it is combined with orange G and acid fuchsin.

**Aniline Red.** See basic fuchsin.

**Archelline 2B.** See Bordeaux red.

**Auramin.** (Syn. *Canary yellow*. *Pyoktaninum aureum*. *Pyoktanin yellow*.) A basic dye of the diphenyl methane series, the only dye of the group concerning which references have been found in the literature on microscopic technique. Its chief use is as a drug, but it has been occasionally employed by the microscopist for some special procedure (p. 189).

**Aurantia.** (Syn. *Imperial yellow*.) An acid dye of the nitro series. It is obsolete as a textile dye and is almost unknown as a biological stain. It is called for, however, in combination with toluidine blue and acid fuchsin in the Champy-Kull technique for demonstrating certain cell constituents (mitochondria, etc.).

**Aurin.** See rosolic acid.

**Aurin R.** See corallin red.

**Azidine Blue 3B.** See trypan blue.

**Azidine Scarlet R.** See vital red.

**Azo-Bordeaux.** See Bordeaux red.

**Azocarmine G.** (Syn. *Azocarmine GX*. *Rosazine*. *Rosinduline GXF*.) A basic azine dye; not an azo-dye as its name would indicate, but actually closely related to the safranins. Employed in tissue staining, especially in the azocarmine modification of the Mallory connective tissue stain.

**Azo-rubin.** See amaranth.

**Azure I.** See methylene azure.

**Azure II.** See Giemsa stain.

**Azure II-Eosin.** See Giemsa stain.

**Azure A.** A lower homologue of methylene blue (a dimethyl instead of the tetramethyl derivative of thionin), a constituent of "azure I" as used in the Giemsa blood stain. At present it is prepared only by the oxidation of methylene blue, and has no commercial use.

It is called for in the latest formula recommended for the tetrachrome blood stain of MacNeal. It proves also of considerable value as a nuclear stain for sections of fixed tissue, especially for delicate procedures where a dye of more definite composition than methylene blue must be employed.

**Azure B.** This basic dye is the trimethyl derivative of thionin and therefore stands between methylene blue and azure A in composition and properties. It is prepared, like azure A, from methylene blue by oxidation and has only recently been obtained in fairly pure form. It is a constituent of "azure I" and is therefore employed in making the Giemsa blood stain. Whether it has any special value as a stain has not yet been determined.

**Azure C.** A basic dye, mono-methyl thionin, prepared at present only by oxidation of methylene blue and not widely available. In composition and properties it stands between thionin and azure A. It has been recommended lately as a nuclear stain for tissues, in combination with eosin and orange II.

**Basic Fuchsin.** See fuchsin, basic.

**Basic Rubin.** See para-fuchsin.

**Benzamine Blue 3B.** See trypan blue.

**Benzo Blue.** See trypan blue.

**Benzo New Blue 2B.** See dianil blue 2R.

**Benzopurpurin 4B.** (Syn. *Cotton red 4B. Dianil red 4C. Diamin red 4B. Sultan 4B. Direct red 4B.*) An acid dye of the azo series that has been used for vital staining, and has occasionally been employed as a protoplasm stain, especially in contrast to hematoxylin.

**Berlin Blue.** See aniline blue, W.S.

**Biebrich Scarlet, Water Sol.** (Syn. *Croceine scarlet, Scarlet B. or EC. Ponceau B. Double scarlet.*) An acid dye, a sulphonated Sudan III. Unlike the latter, it is water soluble and is not, therefore, a fat stain. It has occasionally been used as a protoplasm stain in contrast to methylene blue of hematoxylin.

**Bindschedler's Green.** A basic indamine dye, having some value as an indicator of oxidation-reduction potential.

**Bismarck Brown R.** A dye sometimes sold in place of Bismarck brown Y. Apparently a satisfactory substitute.

**Bismarck Brown Y.** (Syn. *Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown.*) A weakly acid dye of the azo series, formerly quite extensively used as a contrast stain, but now replaced to some extent by other counterstains. It is still used, however, as a mucin stain and is good for vital staining and for staining in bulk. It is employed in staining cellulose walls of plants in contrast to hematoxylin and occasionally for staining bacteria in contrast to gentian violet in the Gram technique. It produces a good stain for photomicrographic purposes and was extensively used by Foot and Strobell<sup>6</sup> for staining smears of Hemipteran germ cells.

**Blood Stains.** See Giemsa stain, Jenner stain, Romanovsky stain, tetrachrome stain, and Wright stain.

**Bordeaux.** See amaranth.

**Bordeaux B, BL, G and R.** See Bordeaux red.

**Bordeaux Red.** (Syn. *Fast red B or P. Cerasin. Archelline 2B. Azo-bordeaux. Acid Bordeaux.*) An acid dye of the azo series, employed as a cytoplasmic stain, particularly when Heidenhain's hematoxylin is to be used immediately afterward as a nuclear stain. Also used in combination

<sup>6</sup>Foot, K., and Strobell, E. C. *Am. J. Anat.*, 4: 199-243, 1905.

with thionin and methyl green, for staining sections, especially of spleen, testis, and liver.

**Bordeaux SF.** See amaranth.

**Brilliant Blue C.** See brilliant cresyl blue.

**Brilliant Congo Red.** See vital red.

**Brilliant Cresyl Blue.** (*Cresyl blue 2N* or *BBS*; *Brilliant blue C.*) A basic dye of the oxazin group, valuable for certain special work on account of its highly metachromatic properties. Its chief biological use is for staining blood to demonstrate the platelets and reticulated corpuscles.

Brilliant cresyl blue is a difficult dye to manufacture and as there is no commercial demand for it, it is expensive. It proves quite a problem from the standpoint of standardization; and although considerably studied of recent years, it is not believed that all batches on the market are yet entirely reliable.

**Brilliant Dianil Red R.** See vital red.

**Brilliant Fat Scarlet B.** See Sudan R.

**Brilliant Green.** (Syn. *Ethyl green*. *Malachite green G.*) A basic dye of the triphenyl methane series. It has little if any use in microscopic technique; but is widely employed by the bacteriologist as a constituent of special culture media, as in the isolation of the typhoid organism and in the search for the colon organism in water. In these media it is used for its selective inhibitory properties on certain microorganisms.

**Brilliant Pink.** See rhodamine B.

**Brilliant Ponceau.** See ponceau 2R.

**Brilliant Purpurin R.** An acid azo dye, that has been employed as a vital stain for yeast.

**Brilliant Yellow S.** (Syn. *Curcumine*; *Yellow WR.*) An acid azo dye, sometimes employed as a counterstain in a modification of the Ziehl technique for demonstrating the tubercle organism.

**Brom Chlor Phenol Blue.** An acid dye of the sulphonephthalein series, changing from yellow to blue through the H-ion range between 3.0 and 4.6, and hence having use as an indicator. May also be used as an indicator of reaction in vital staining.

**Brom Cresol Green.** An acid dye of the sulphonephthalein series, used as an indicator, having a sensitive range between pH 3.8 and 5.4. This range is close to that of methyl red, and as the dye is not subject to reduction and fading of its color, it is often preferred to methyl red. Can be employed in microscopic work as indicator of reaction when used for vital staining.

**Brom Cresol Purple.** An acid dye of the sulphonephthalein series, much used as an indicator of reaction, having its sensitive range a little

to the acid side of neutrality, namely between pH 5.2 and 6.8. For certain accurate chemical work it is undesirable because it is blue in reflected light but purple in transmitted light (in such cases substitute brom phenol red); but this dichromatism does not interfere with most of its biological uses. In vital staining, it also serves as an indicator of reaction.

**Brom Phenol Blue.** An acid dye of the sulphonephthalein series, employed as an indicator, with a sensitive range from pH 3.0 to 4.6. In vital staining it finds employment as an indicator of reaction.

**Brom Phenol Red.** An acid dye of the sulphonephthalein series, having indicator properties, with a sensitive range between pH 5.2 and 6.8. Can be used as an indicator of reaction in vital staining. For accurate chemical work it is recommended in place of brom cresol purple, because it is free from the dichromatism which is sometimes troublesome in the latter.

**Brom Thymol Blue.** An acid dye of the sulphonephthalein series, one of the most useful indicators of reaction, as its sensitive range includes the neutral point, covering pH 6.0 to 7.6. In vital staining it also serves as an indicator of reaction.

**Brown Salt R.** See chryosidin Y.

**Buffalo Garnet H.** See Erie garnet B.

**Caesar Red.** See eosin, bluish.

**Canary Yellow.** See auramin.

**Carmoisine.** See chromotrope 2R.

**Celestin Blue B.** (Syn. *Coreine* 2R.) A basic dye of the oxazin series, used in the form of an iron lake, as a nuclear stain.

**Cerasin.** See Bordeaux red.

**Cerasin Red.** See Sudan III.

**China Blue.** See aniline blue, W.S.

**Chlor Cresol Green.** An acid dye of the sulphonephthalein series, having indicator properties, with a sensitive range between pH 4.0 and 5.6. May be used as an indicator of reaction in vital staining. Is frequently to be substituted for methyl red, when the unstable nature of the latter is objectionable.

**Chlor Phenol Red.** An acid dye of the sulphonephthalein series employed as an indicator, with a sensitive range between pH 4.8 and 6.4. Can occasionally be substituted for methyl red, where the unstable nature of the latter renders it undesirable. May also find employment in vital staining, where it can serve as an indicator within the tissue.

**Chlorazol Blue 3B.** See trypan blue.

**Chrom Blue GCB.** See gallocyanin.

**Chrom Violet.** An acid dye of the rosolic acid series.

**Chromotrope 2R.** (Syn. *Chromotrope N2R*. *Chromotrope blue* 2R.)

*XL carmoisine 6R. Fast fuchsin G. Acid phloxine GR.*) An azo dye, closely related to orange G, which has been employed as a counterstain in histology.

**Chrysoidin Y.** (Syn. *Brown salt R. Dark brown salt R.*) A basic azo dye. Occasionally used as a substitute for Bismarck brown.

**Congo.** See Congo red.

**Congo Blue 3B.** See trypan blue.

**Congo Corinth G or GW.** See Erie garnet B.

**Congo Red.** (Syn. *Congo. Cotton red. Direct red.*) An acid dye of the azo group, best known to the biologist as an indicator. It also has certain value as a histological stain, as for demonstrating axis cylinders in nervous tissue, and mucin in plants, as well as serving for a general background stain in contrast to hematoxylin, and other nuclear dyes.

**Corallin Red.** (Syn. *Aurin R.*) A compound dye, assumed to be the pararosanolin salt of pararosolic acid.

**Corallin Yellow.** The sodium salt of rosolic acid.

**Coreine 2R.** See celestin blue B.

**Corinth Brown G.** See Erie garnet B.

**Cotton Blue.** See methyl blue.

**Cotton Corinth G.** See Erie garnet B.

**Cotton Red.** See Congo red.

**Cotton Red 4B.** See benzopurpurin 4B.

**Cresol Red.** An acid dye of the sulphonephthalein series, used as an indicator, having a sensitive range between pH 7.2 and 8.8. In vital staining it also serves as an indicator of reaction.

**Cresolphthalein.** A weakly acid dye of the xanthene series, very closely related to the much better known phenolphthalein. Like the latter, it is employed as an indicator, and has a very similar range (pH 8.3 to 10.0). For some purposes it is preferable to phenolphthalein.

**Cresyl Blue 2RN or BBS.** See brilliant cresyl blue.

**Cresylecht Violet.** See cresyl violet.

**Cresyl Violet.** (Syn. *Cresylecht violet*, i.e., Cresyl fast violet.) A basic dye of the oxazin group. It is not a textile dye, and is not widely used as a stain; but finds some employment because of its strongly metachromatic properties. According to Ehrlich, it stains nuclei violet, plasma blue, amyloid, mucin, and mast cell granules red. It is valuable for making permanent preparations of nervous tissue. It has recently been employed for staining sections of fresh tumor tissue in diagnostic and autopsy work.

At least two different dyes have been used in the past by biologists under this name and some confusion has resulted. There is no question as to which is correctly termed cresyl violet, but it is doubtful whether

in any given instance this is the dye actually used by the author of some technique calling for a stain so named. The matter is still under investigation.

**Croceine Scarlet.** See Biebrich scarlet, water sol.

**Crystal Violet.** (Syn. *Violet C, G* or *7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.*) A basic dye of the triphenyl methane group—hexamethyl pararosaniline. It is a constituent of all the bluer shades of methyl violet and gentian violet; and apparently the most important constituent of pre-war gentian violet for the purposes for which the microscopist employed the latter (see gentian violet). Crystal violet has been definitely specified in certain procedures, such as Benda's crystal-violet-alizarin method for staining chondriosomes, and in a more recently proposed stain (Jackson<sup>7</sup>) in combination with erythrosin for staining lightly lignified walls in plants. Besides such special procedures as this, it is now highly recommended in nearly all procedures calling for gentian violet, as crystal violet is a definite chemical compound and therefore more constant in composition than gentian violet. The only exception to this is that in certain procedures crystal violet is slightly too blue for the proper contrast; in such instances one of the methyl violets (2B or redder) is to be recommended (see methyl violet).

**Curcumine.** See brilliant yellow S.

**Dahlia.** See Hofmann violet.

**Dahlia B.** See methyl violet.

**Dark Brown Salt R.** See chrysoidin Y.

**Diamine Bordeaux CGN.** See Erie garnet B.

**Diamine Red 4B.** See benzopurpurin 4B.

**Diamond Fuchsin.** See basic fuchsin.

**Diamond Green.** See malachite green.

**Dianil Blue 2R.** (Syn. *Direct steel blue BB. Benzo new blue 2B. Naphthamine brilliant blue 2R.*) An azo dye closely related to trypan red and trypan blue; sometimes employed in vital staining.

**Dianil Blue H<sub>3</sub>G.** See trypan blue.

**Dianil Red 4C.** See benzopurpurin 4B.

**Dianthin B.** See erythrosin, bluish.

**Dianthin G.** See eosin, bluish.

**Diazin Green.** See Janus green B.

**Direct Garnet R.** See Erie garnet B.

**Direct Red.** See Congo red.

**Direct Red 4B.** See benzopurpurin 4B.

**Direct Steel Blue BB.** See dianil blue 2R.

**Direct Violet C.** See Erie garnet B.

<sup>7</sup> Jackson, G. *Stain Technology*, 1: 33, 1936.



**Double Green.** See methyl green.

**Double Scarlet.** See Biebrich scarlet, water sol.

**Emerald Green.** See malachite green.

**Eosin, Alcohol Sol.** See methyl eosin and ethyl eosin.

**Eosin, Bluish.** (Syn. *Eosin*, *BN*, *B*, *BW*, or *DKV*. *Safrosin*. *Eosin scarlet B* or *BB*. *Scarlet J*, *JJ*, or *V. Nopalin G. Caesar red*.) An acid dye of the xanthene series, dinitro-dibrom fluorescein. It proves to be a useful counterstain for hematoxylin. Ordinarily, however, if a shade deeper than eosin Y is desired, better results can be obtained with erythrosin, phloxine or rose bengal than with eosin B. One company, in fact, puts on the market a product labelled "eosin, bluish blend," (formerly sold as eosin bluish) which is a mixture of eosin Y with some one of the dyes just named; it is very satisfactory for certain staining procedures, but must not be confused with true eosin B.

**Eosin BN, B, BW, and DKV.** See eosin, bluish.

**Eosin J.** See erythrosin, bluish.

**Eosin S.** See ethyl eosin.

**Eosin Scarlet B and BB.** See eosin bluish.

**Eosin W or WS.** See eosin Y.

**Eosin Y.** (Syn. *Water sol. eosin*. *Eosin W* or *WS*.) An acid dye of the xanthene group—tetrabrom fluorescein. There are several other eosins besides eosin Y, all derivatives of fluorescein, but this grade (i.e., yellowish eosin) is ordinarily referred to when eosin alone is specified.

Eosin Y is probably the most commonly used of the red acid dyes. It is one of the most valuable cytoplasm stains known and is employed in a great variety of procedures. It is most frequently used as a counterstain for hematoxylin and the green or blue basic dyes; also by Mann, mixed with methyl blue, as a tissue stain. One of the uses for which it is at present in greatest demand is in the various blood stains, in which it is combined with methylene blue or with some oxidation product of the latter (see Giemsa stain, Jenner stain, Romanovsky stain, tetrachrome stain, Wright stain).

**Erie Garnet B.** (Syn. *Congo corinth G* or *GW*. *Corinth brown G*. *Cotton corinth G*. *Amanil garnet H*. *Direct garnet R*. *Buffalo garnet H*. *Direct violet C*. *Diamine Bordeaux CGN*.) An acid azo dye, that has been employed, mixed with azure A, for staining fresh frozen tissue.

**Erythrosin B.** See erythrosin, bluish.

**Erythrosin BB.** See phloxine.

**Erythrosin, Bluish.** (Syn. *Erythrosin B*. *Pyrosin B*. *Eosin J*. *Iodo-eosin B*. *Dianthin B*.) An acid dye of the xanthene group—tetra-iodo fluorescein. This is probably the dye that has been used in the past in instances where "erythrosin" is specified. No procedures are definitely

known, in fact, where the yellowish erythrosin is more satisfactory than the bluish type; and as the former is an obsolete dye, while the latter is a recognized food color, the bluish type is the one now ordinarily obtainable.

Erythrosin is used somewhat as an indicator; and has fairly extensive employment as an histological stain, although not so widely used as eosin Y. Its value for histological purposes is as a red counterstain not quite so yellow and not quite so strongly acid in character as eosin Y. These two properties make it useful in some procedures where eosin Y is not wholly satisfactory. It has been employed as a stain for bacteria in soil.

**Erythrosin R or G.** See erythrosin, yellowish.

**Erythrosin, Yellowish.** (Syn. *Erythrosin R* or *G.* *Pyrosin J.* *Dianthin G.* *Iodo-eosin G.*) An acid dye of the xanthene group—di-iodo fluorescein. It may have been used in the past for some procedures in which simply "erythrosin" has been named. The bluish grade is now ordinarily employed, however.

**Ethyl Eosin.** (Syn. *Eosin, alcohol sol.* *Eosin S.*) An acid dye of the xanthene group, the ethyl ester of eosin Y. This dye has ordinarily been sold in the past as alcohol soluble eosin; but the term ethyl eosin is to be preferred as there are other alcohol soluble eosins, particularly the one known as methyl eosin. Apparently the ethyl compound is the one that was available before the war. Since the war there has been some confusion in the matter.

It is a very valuable counterstain after Delafield's hematoxylin in general histological work. It is specially called for in contrast to methylene blue in searching for Negri bodies in the nervous tissue of animals suspected of having had rabies.

**Ethyl Green.** See brilliant green. Also the name of one type of methyl green (q.v.).

**Ethyl Purple 6B.** See ethyl violet.

**Ethyl Violet.** (Syn. *Ethyl purple 6B.*) A rosanilin dye, closely related to methyl violet. Has been employed in combination with Biebrich scarlet to stain the islets of Langerhans.

**Excelsior Brown.** See Bismarck brown Y.

**Fast Acid Blue R.** (Almost the same as *Violamine 3B.*) A complex rhodamine, that has been used for staining bacteria in soil.

**Fast Acid Green N.** See light green SF, yellowish.

**Fast Blue B.** See naphthol blue.

**Fast Blue 3R.** See naphthol blue.

**Fast Fuchsin G.** See chromotrope 2R.

**Fast Green FCF.** An acid dye of the di-amino tri-phenyl methane

series, closely related to light green SF yellowish. It is now preferred to the latter as a counterstain because of its greater permanency.

**Fast Printing Green.** See naphthol green Y.

**Fast Red.** See amaranth.

**Fast Red B or P.** See Bordeaux red.

**Fast Violet.** See gallocyanin.

**Fast Yellow.** An acid dye of the azo group closely related to methyl orange, rarely employed as a biological stain, although occasionally called for in certain special procedures.

**Fettponceau.** See Sudan IV.

**Fluorescein.** (Syn. *Uranin*.) An acid dye, the simplest member of the fluorane group and the mother substance of the eosins. It is of low tinctorial power, but has a very marked yellow fluorescence which is detectable even in extremely great dilution. The dye has no apparent value as a biological stain.

**Fuchsin, Acid.** (Syn. *Fuchsin S, SN, SS, ST, or S III. Acid magenta. Acid rubin*.) An acid dye (or group of dyes) of the trimethyl methane series, derived from basic fuchsin by sulphonation. As there are four primary basic fuchsins theoretically possible, and as each one of them may yield a mono-, di- or trisulphonic acid, an extremely great variety of different acid fuchsins may occur, and its manufacture is very difficult to control so as to produce a uniform product. Recent work by Scanlan, Holmes and French,<sup>8</sup> however, shows that to a certain extent such control is possible, and the future may see a more uniform product supplied to microscopists.

Acid fuchsin is one of the most widely used cytoplasm stains. It has also been called for in many special procedures, such as the Van Gieson (p. 405) connective tissue stain, in which it is used with picric acid after hematoxylin to differentiate smooth muscle from connective tissue; the Ehrlich-Biondi stain, in which with methyl green and orange G it is employed in histology and for staining blood smears; the Ehrlich tri-acid stain for blood, in which it is combined with orange G and methyl green; the Pianese stain for cancer tissue (with malachite green and martius yellow), now mostly used in studying infected vascular plants; as a constituent of the Mallory aniline blue connective tissue stain; and with methyl green as a stain for mitochondria.

**Fuchsin, Basic.** (Syn. *Diamond fuchsin. Magenta. Rubin. Aniline red*.) A basic dye (or group of dyes) of the triphenyl methane group. There are four primary basic fuchsins theoretically possible; according to the number of methyl groups introduced into the benzene rings. If no such methyl group is present the compound is known as pararosanilin;

<sup>8</sup> Scanlan, Holmes, and French, *Stain Technology*, 2: 50, 1927.

if one, it is known as rosanilin; if three, as new fuchsin. All three of these compounds are known in fairly pure form. The compound intermediate between rosanilin and new fuchsin, although possible and undoubtedly present in some of the basic fuchsin, is not obtained pure without special methods of preparation, and is not a recognized dye. Ordinary basic fuchsin is mixtures of pararosanilin and rosanilin.

Basic fuchsin is one of the most powerful nuclear dyes, almost too powerful for delicate histological work, on account of its tendency to overstain. It is employed for mucin and elastic tissue; but by far its most important microscopic use is as a bacterial stain, particularly in the Ziehl-Neelsen method for differentiating the tubercle organism and thus diagnosing tuberculosis. The bacteriologist also uses it, in decolorized form, in the Endo medium as an indicator to distinguish between the typhoid and colon organisms, thanks to the property of the latter of restoring the red color if growing in the presence of lactose.

Basic fuchsin, decolorized by sulphite as in the Endo medium, is employed by chemists as an indicator for detecting aldehyde, in the presence of which a violet-red color is produced. It is similarly used in the Feulgen stain, in which it has been thought to act as a microchemical reagent to show the presence of aldehyde-like bodies in nuclei. Its exact significance in this procedure, however, has not yet been determined. Not all basic fuchsin are satisfactory in the Feulgen procedure.

**Fuchsin NB.** See new fuchsin.

**Fuchsin S, SN, SS, ST, or S III.** See acid fuchsin.

**Gallamin Blue.** A basic dye of the oxazin series, sometimes used as a nuclear stain.

**Gallocyanin.** (Syn. *Alizarin blue RBN. Chrom blue GCB. Fast violet*). A basic oxazin dye, sometimes used as a nuclear stain.

**Gambine.** See naphthol green Y.

**Gentian Blue.** See spirit blue.

**Gentian Violet.** This is not a textile dye, and is not recognized in the regular dye industry. Under this name, Grüber sold to biologists a mixture of one of the higher methyl violets with crystal violet. It may therefore be regarded as a very highly methylated methyl violet. Soon after the war it was found that some dealers in the United States were selling crystal violet under the name of gentian violet, and as this was found to give better results than most of the methyl violets, the practice has been recognized by the adoption of the following definition of gentian violet by the Commission on Standardization of Biological Stains:

"Gentian violet . . . must be either pentamethyl or hexamethyl pararosanilin, or else a mixture of methylated pararosanilins composed

primarily of the compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B."

The Commission is at present recognizing two grades of gentian violet: one gentian violet bluish, which is primarily or entirely crystal violet; the other gentian violet reddish, which is apparently a mixture, one of the deeper methyl violets.

This stain is of chief value to the biologist as a nuclear or chromatin stain, having many histological and cytological applications, the one for which it is most commonly employed at present being the Flemming triple stain (with orange G and safranin). It is also used for various histological procedures. It is an extremely valuable bacterial stain, especially in the Gram technique, with its various modifications. Recently it has been further employed for its inhibiting effect on certain bacteria and has been suggested as a therapeutic agent.

For the Gram stain (as well as for staining bacteria in general) the bluish grade or crystal violet proves most satisfactory; this grade is likewise to be preferred in most histological and cytological work, including the Flemming technique. In certain variations of the Flemming stain, however, some microscopists seem to get better results with the reddish grade. It has also been stated that crystal violet alone does not have such pronounced inhibiting action on bacteria as do some of the mixtures that have been sold as gentian violet.

**Giemsa Stain.** (Syn. *Azure-II-eosin*.) A so-called "neutral stain," used in staining blood; a compound of eosin with one of the derivatives of methylene blue, known as "azure II." Azure II is a mixture of azure I (see methylene azure) and methylene blue in equal parts. In the Giemsa stain this mixture is compounded with eosin, and the eosinates which are formed, being practically insoluble in water, precipitate out. This precipitate is the Giemsa stain, and can be used in alcoholic solution, as are the Wright stain and tetrachrome stain.

**Gold Orange.** See orange II.

**Gray R, B, BB.** See nigrosin, water sol.

**Green PL.** See naphthol green B.

**Helianthin.** See methyl orange.

**Heliotrope B.** See amethyst violet.

**Helvetia Blue.** See methyl blue.

**Hexamethyl Violet.** See crystal violet.

**Hofmann Green.** See iodine green.

**Hofmann Violet.** (Syn. *Dahlia*. *Iodine violet*. *Red violet*. *Violet R*, *RR*, or *4RN*.) A basic dye of the triphenyl-methane series, closely related to methyl violet, but having ethylated instead of methylated amino

groups. Like methyl violet it may vary considerably in shade, as it is a mixture of various homologues of the series.

The dye has been used occasionally in histological work, and has been specially recommended for staining mast cells and amyliod. It has considerable metachromatic effect.

**Imperial Yellow.** See aurantia.

**Indian Blue 3RD.** See naphthol blue.

**Indigo.** (Syn. *Indigo blue*.) A well-known blue dye formerly obtained from certain species of plants, but now ordinarily manufactured synthetically. It was formerly employed to some extent as a biological stain, but has little such use at the present time.

**Indigo Blue.** See indigo.

**Indigo Carmine.** (Syn. *Indigotine IA*.) A blue dye closely related to indigo, which is sometimes used as a cytoplasm stain in contrast to carmine.

**Indigotine IA.** See indigo carmine.

**Indin Blue 2RD.** See naphthol blue.

**Indophenol Blue.** A colored compound of the indophenol group; possibly the same indophenol as employed by Herxheimer as a fat stain.

**Indulin, Spirit Soluble, and Indulin, Water Soluble.** Two azin dyes, closely related to the safranins; occasionally employed as a counter-stain for tissue in bulk, or for the negative staining of bacteria.

**Indulin Black.** See nigrosin, water sol.

**Iodo-eosin B.** See erythrosin, bluish.

**Iodo-eosin G.** See eosin, bluish.

**Iodine Green.** (Syn. *Hofmann green*.) A basic dye of the triphenyl-methane series, closely related to methyl green, differing from the latter in being a rosanilin derivative while the latter is supposed to be derived primarily from pararosanilin. It is a nuclear or chromatin stain having selective properties that make it of value in certain special procedures. It has been used for staining nervous tissue in combination with acid fuchsin and picric acid, and as a blood stain in combination with acid fuchsin; with basic fuchsin it has been employed for staining chromatin in plant tissue, and with acid fuchsin for lignified xylem. It is also employed for staining mucin and amyloid, having the property of giving the latter a red instead of a green color.

**Iodine Violet.** See Hofmann violet.

**Iris Violet.** See amethyst violet.

**Isamine Blue.** A complex sulphonated rosanilin that has occasional use as a vital dye.

**Isorubin.** See new fuchsin.

**Janus Green B.** (Syn. *Diazin green*.) A basic dye related both to the

safranins and to the azo dyes. It is best known to the biologist for its use in demonstrating chondriosomes, stained *intra vitam*. It has also been employed with neutral red for staining sections of embryos.

**Jenner Stain.** One of the first compounds of eosin and methylene blue used for staining blood. It is still employed to some extent, although for most purposes it gives much less satisfactory results than do the Geimsa, the Wright or the tetrachrome stains (q.v.). Jenner's stain is merely the eosinate of methylene blue, precipitated from aqueous solution and redissolved in methyl alcohol. As polychrome methylene blue is not used in preparing it, the stain does not give the many colored effects obtained with any one of the other three blood stains just mentioned.

**Lacmoid.** An indicator, closely related to resorcin blue (q.v.).

**Lake Ponceau.** See ponceau 2R.

**Lauth's Violet.** See thionin.

**Leather Brown.** See Bismarck brown Y.

**Leather Yellow.** See phosphine.

**Light Green SF, Yellowish.** (Syn. *Light green 2G, 3G, 4G, or 2GN. Acid green. Fast acid green N.*) An acid dye of the triphenylmethane series. It is a valuable cytoplasm stain often employed for staining tissues in contrast to iron hematoxylin, although it fades badly if exposed to bright light. It has also been used in contrast to safranin as a cytoplasm stain for spermatozoa, and as a cytoplasm and cellulose stain in plant histology.

Care must be taken not to confuse this dye with other light greens (such as malachite green and methyl green). If the grade "SF yellowish" is not specified in ordering there is danger of obtaining some other dye than the one desired.

**Light Green 2G, 3G, 4G, or 2Gn.** See light green SF, yellowish.

**Light Green N.** See malachite green.

**Litmus.** A natural dye obtained from certain lichens. It is used chiefly as an indicator; and at present much less even for that purpose than formerly.

**Lyons Blue.** See spirit blue.

**Magdala Red.** (Syn. *Naphthaline red. Naphthaline pink. Naphthylamine pink. Sudan red.*) A basic dye of the safranin group (a naphthosafranin). True magdala red is very expensive, and was sold before the war under the name "magdala rot, echt." A cheaper "magdala red," without qualifying designation, was also put on the market by Grüber before the war, which proves to have been (in some instances, at least) a fluorescein derivative (phloxine or erythrosin) rather than a safranin. It is possible to obtain a fluorescein dye of exactly the shade of magdala red, indistinguishable from it even by spectrophotometric analysis, but it

has acid instead of basic properties. Such seems to have been the nature of the cheaper "magdala red" available before the war. At least one instance is known in which this has led to confusion. Chamberlain<sup>9</sup> has employed "magdala red" with aniline blue in staining algae; but finds that true magdala red is unsatisfactory in the technique, while phloxine gives him more dependable results than obtained with the so-called "magdala red" which he formerly used. Magdala red has also been employed occasionally in animal histology, either as a nuclear or an elastin stain; for these purposes probably the true dye was used.

**Magenta.** See basic fuchsin.

**Malachite Green.** (Syn. *Emerald green. New Victoria green. Diamond green. Solid green. Light green N.*) A weakly basic dye of the triphenyl methane series. It was formerly employed for various histological purposes, but today has largely been replaced by methyl green. It is still used by botanists (together with acid fuchsin and martius yellow) for staining host tissue in plants infected with fungi.

Although this dye is also known as light green N, it is not the dye which the microscopist wants in procedures calling for light green. Light green SF yellowish is the dye ordinarily to be employed in such procedures, but when light green, without qualifying designation, is ordered, the dye manufacturer sometimes furnishes malachite green.

**Malachite Green G.** See brilliant green.

**Manchester Brown.** See Bismarck brown Y.

**Manchester Yellow.** See Martius yellow.

**Mandarin G.** See orange II.

**Marine Blue.** See aniline blue, W.S.

**Martius Yellow.** (Syn. *Manchester yellow. Naphthol yellow.*) An acid dye of the nitro series, quite closely related to picric acid. It was used by Pianese in combination with malachite green and acid fuchsin for studying cancer tissue; the same technique is now applied by botanists to the study of sections of plants infected by fungi. The dye is also employed in preparing certain light filters for photomicrography.

**Meldola's Blue.** See naphthol blue.

**Mercurochrome 220.** A mercuric derivative of fluorescein, closely related to eosin. Primarily employed as a weak antiseptic, but occasionally called for in place of eosin or erythrosin as a cytoplasm stain.

**Meta-cresol Purple.** An acid dye of the sulphonaphthalein series, used for its indicator properties, changing from red to yellow through the range between pH 1.2 and 2.8, and from yellow to purple between pH 7.4 and 9.0. Can be employed in microscopic work as an indicator of reaction when used as a vital stain.

<sup>9</sup> Chamberlain, C. J. *Stain Technology*, 2: 91, 1927.



**Metanil Yellow.** (Syn. *Orange MNO* or *MN. Acid yellow R. Soluble yellow OL. Yellow M. Trapaeolin G.*) An acid dye of the azo series, employed in the Masson trichrome technique.

**Methyl Blue.** (Syn. *Cotton blue. Helvetia blue.*) An acid dye of the triphenyl methane series, it is one of the components of aniline blue, water soluble (q. v.). It is largely employed in the Mann (p. 536) technique (with eosin) for staining nerve cells. It is a good counterstain, for contrast to a red nuclear stain such as safranin or carmine; picric acid is sometimes used with it when it is thus employed.

**Methyl Eosin.** (Syn. *Eosin, alcohol sol.*) An acid dye of the xanthene group, the methyl ester of eosin Y. It may sometimes have been employed where eosin, alcohol soluble, has been specified, although for this purpose the dye most frequently used has probably been ethyl eosin (q. v.).

**Methyl Green.** (Syn. *Double green. Light green.*) A basic dye of the triphenylmethane series, closely related to iodine green, derived from crystal violet by the introduction of a seventh methyl group through the action of methyl chloride or iodide. Sometimes an ethyl halide is thus used in its preparation; in which case the dye may be designated *ethyl green*. It is readily decomposed and this added methyl or ethyl group driven off. Thus some of the violet dye from which it is derived is almost sure to be present; and this fact partly, at least, explains the metachromatic action of methyl green.

Methyl green is an extremely valuable nuclear stain in histology and chromatin stain in cytology, and is used with various counterstains especially acid fuchsin. Ehrlich has combined it to form a "neutral stain" with orange G and acid fuchsin, and employs the compound as a blood stain. Botanists find it valuable for lignified xylem, with acid fuchsin for contrast. It is much used today mixed with pyronin in the Pappenheim stain, employed for staining the gonococcus and mast cells. It is a very valuable chromatin stain for protozoa.

Following the war, methyl green was one of the most difficult stains to obtain in satisfactory quality. The problem seems to have been solved completely now, however, and for some time no complaints of American methyl greens have been heard.

**Methyl Orange.** (Syn. *Orange III. Helianthin. Gold orange. Tropaeolin D.*) A weakly acid dye of the azo series, chiefly employed as an indicator. It is occasionally used in histology, however, as a counterstain and in one or two special procedures.

**Methyl Red.** An old and well known indicator, having a sensitive range between pH 4.4 and 6.0. Where a quick reading is desired it is excellent but the color fades rapidly. Hence it is not suitable for the

preparation of color standards, nor for work where it is desired to study H-ion changes in a solution to which an indicator is added. For such purposes it should be replaced by one of the sulphonephthalein indicators—either brom cresol green or chlor cresol green if the acid half of its range is under consideration, or chlor phenol red in the case of the alkaline end of the range.

**Methyl Violet.** (Syn. *Dahlia B. Paris violet. Pyoktanin blue. Gentian violet.*) A basic dye of the triphenyl methane series. (See also gentian violet and crystal violet.) It is a mixture of tetramethyl, pentamethyl, and hexamethyl rosanilins or pararosanilins. The hexamethyl compound alone is known as crystal violet. The greater the degree of methylation the less red and the more blue is its shade; this leads to various shade designations such as 2R, R, B, 2B, 5B, 6B, etc. (R standing for red, B for blue). Each designation, such as *methyl violet 2B*, indicates a fairly definite shade, but not a definite chemical composition, as the same shade may be obtained by different mixtures of compounds in this group.

Methyl violet, sometimes with and sometimes without shade designation, has been specified in various procedures as a nuclear stain and sometimes as a bacterial stain in the Gram technique. For some of these procedures, as good (or better) results can be obtained with crystal violet alone as with one of the indefinite mixtures known as methyl violet; in such cases, crystal violet should be used, and specified for the technique in question, because it is a definitely known chemical compound. In some instances, however, crystal violet is not sufficiently reddish in shade to give the proper contrast; then a methyl violet should be used. For such purposes the grade 2B seems fairly satisfactory; it is distinctly redder than crystal violet and yet not so red but that it will show contrast to a red counterstain.

**Methyl Violet 10B.** See crystal violet.

**Methylene Azure.** (Syn. *Azure I.*) A name given to an oxidation product of methylene blue. It is of secret composition but apparently consists partly of azure A and partly of azure B. It is called for in the Giemsa stain for blood (q. v.) and was originally specified in the tetrachrome stain. More recent formulae of the latter, however, call for azure A instead of the mixture formerly known as azure I (see tetrachrome stain). Apparently in most instances where methylene azure is called for, azure A can be used to best advantage.

**Methylene Blue.** (Syn. *Swiss blue.*) A basic dye of the thiazin series, tetramethyl thionin. To the pathologist and bacteriologist methylene blue is perhaps the most valuable of all the stains, while the zoologist also finds many uses for it. It is used, first, as a nuclear stain in histology;

second, as a bacterial stain, notably in milk work and in the diagnosis of diphtheria; third, in the vital staining of nervous tissue and fourth, in combination with eosin in the blood stains.

Its greatest value along these lines is that it is a strong basic dye without great tendency to overstain, and that it has very pronounced metachromatic properties. These metachromatic properties are especially likely to develop in methylene blue solutions on standing, and such a solution is known as "polychrome methylene blue." Its formation is hastened by boiling with alkali. What actually happens is that the methylene blue is partly oxidized into its lower homologues, methylene violet and the azures (q. v.). In preparing Wright's stain, methylene blue is polychromed before combining with eosin.

The ease with which methylene blue oxidizes into these lower homologues makes it almost impossible to secure a pure methylene blue. Practically all, if not all, commercial methylene blues are partly polychromed, and it has been claimed that the most valuable properties of methylene blue are the oxidation products thus produced. It appears, in fact, that pure methylene blue is a relatively poor nuclear stain, with very little metachromasy, while the lower homologues are successively better nuclear stains and more highly metachromatic.

Before the war some of the methylene blue sold to biologists was in the form of the zinc double salt. This form is quite insoluble in alcohol and is a comparatively poor stain. For all staining purposes investigated, better results can be obtained with the medicinal grade of methylene blue, which according to the U.S.P. requirements must be zinc-free. It is the form in which methylene blue is now ordinarily furnished to biologists in America.

**Methylene Blue NN.** See new methylene blue N.

**Methylene Blue O.** See toluidine blue O.

**Methylene Green.** A nitro derivative of methylene blue. It is occasionally used as a substitute for methyl green, especially by botanists in the case of wood and fixed chromatin; it gives good results in combination with eosin.

**Methylene Violet.** Bernthsen. This is not a textile dye, and must be distinguished from methylene violet, RRA or 3RA which is better known commercially. It is one of the oxidation products of methylene blue. It plays an important part in the nuclear and granule staining of polychrome methylene blue. A definite quantity of this dye is mixed with methylene blue, azure A, and eosin in the tetrachrome blood stain of MacNeal, p. 325.

**Naphthaline Pink.** See magdala red.

**Naphthaline R.** See naphthol blue.

**Naphthaline Red.** See magdala red.

**Naphthamine Blue, 3BX.** See trypan blue.

**Naphthol Blue.** (Syn. *New blue B. Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.*) A basic dye of the oxazin group, which has been occasionally employed as a vital stain.

**Naphthol Green Y.** (Syn. *Fast printing green. Gambine*) and **Naphthol Green B.** (Syn. *Naphthol green. Green PL. Acid green O.*) Two nitro dyes, one or the other of which has been employed as an histological counterstain.

**Naphthol Red.** See amaranth.

**Naphthol Yellow.** See Martius yellow.

**Naphthylamine Pink.** See magdala red.

**Narcein.** An acid dye of the azo series, closely related to orange II. It has very little use as a stain, but has been employed in combination with pyronin and methyl green or methylene blue to form a compound dye.

**Neutral Red.** A weakly basic dye of the azin series, ordinarily the chloride of toluylene red. It has recently been proposed to employ the iodide, however, as it is claimed to be more easily purified than the chloride.

Neutral red is best known as an indicator, but has special staining value where a weakly basic, non-toxic dye is called for. It has been employed as a vital nuclear stain; for the staining of fresh blood or pus in a moist chamber; for the vital staining of protozoa; for bringing out Nissl granules in nerve cells; and it has some use in general histological staining, especially for embryological tissue in combination with Janus green.

**Neutral Violet.** A weakly basic dye of the azin group, closely related to neutral red and having very similar properties. It has rarely been used in microscopic work.

**New Blue B.** See naphthol blue.

**New Fuchsin.** (Syn. *Isorubin. Fuchsin NB.*) A basic dye of the triphenyl methane series. It differs from rosanilin in that three methyl groups instead of one are attached to the benzene rings. It is seldom recommended as a stain, but basic fuchsins containing a large proportion of it have been found to be splendid stains for certain purposes.

**New Methylene Blue N.** (Syn. *Methylene blue NN.*) A basic dye of the thiazin group, of somewhat greener shade than true methylene blue. It apparently was present in certain pre-war lots of methylene blue; and it has been found that in at least one instance results carried on with pre-war "methylene blue" can be duplicated with new methylene blue, but not with true methylene blue.

**New Pink.** See phloxine.

**New Ponceau 4R.** See ponceau 2R.

**New Victoria Green.** See malachite green.

**Niagara Blue 3B.** See trypan blue.

**Night Blue.** See spirit blue.

**Nigrosin, Water Sol.** (Syn. *Nigrosin W*, *WL*, etc. *Gray R*, *B*, *BB*. *Silver gray*. *Steel gray*. *Indulin black*.) A basic dye of poorly understood composition. It is apparently a mixture of an indulin (violet in color) with a yellow dye so as to make an almost black solution. It has been recommended for staining various kinds of tissue, either alone or in combination with other stains such as hematoxylin. It is employed in studying algae and fungi. One of its most striking uses is in the Dorner spore stain for bacteria, in which technique it has the property of withdrawing basic fuchsin from the vegetative portions of the cells but not from the spores. But as it itself does not stain the cells, they stand out colorless in a gray background, while the spores retain the fuchsin.

**Nile Blue A.** See Nile blue sulphate.

**Nile Blue Sulphate.** (Syn. *Nile blue A*.) A basic dye of the oxazin series, closely related to brilliant cresyl blue. The use for which this dye is best known to the biologist is the Lorrain Smith fat stain. In this procedure, the dye is boiled with dilute sulphuric acid and is thus partly converted into a new dye of the class known as oxazones. This oxazone dye is red and is fat-soluble; while Nile blue itself is not fat-soluble, but combines readily with fatty acids. As a result, the technique serves to distinguish between the free fatty acids in histological material and the neutralized fats, the former staining blue, the latter red.

**Nitrazine Yellow.** A colored azo compound recently recommended as an indicator; can be employed absorbed on filter paper slips, like litmus.

**Nopalin G.** See eosin, bluish.

**Oil Red.** See Sudan III.

**Oil Red IV.** See Sudan IV.

**Oil Red O.** (Syn. *Oil scarlet*, *Sudan II*, *X*, or *AX*). A fat-soluble dye of the azo group. It has not until recently been employed for microscopic work; but has been recommended,<sup>10</sup> for staining fat in sections.

**Oil Scarlet.** See oil red O.

**Oil Vermilion.** See Sudan R.

**Orange II.** (Syn. *Gold orange*. *Orange A*, *P* or *R*. *Acid orange*. *Orange extra*. *Mandarin G*. *Tropeolin OOO No. 2*.) An acid dye of the

<sup>10</sup> French, R. W. *Stain Technology*, 1: 78, 1926.

Proescher, F. *Stain Technology*, 2: 60, 1927.

azo series. It has some value as a counterstain, having been recommended by French for use with azure C and eosin as a tissue stain.

**Orange III.** See methyl orange.

**Orange A, P, or R.** See orange II.

**Orange Extra.** See orange II.

**Orange G.** (Syn. *Wool orange 2G*.) An acid dye of the azo series. It is one of the most valuable histological counterstains, and is the orange dye most frequently used by histologists. It is employed with various nuclear stains, but special mention should be made of safranin and gentian violet (with which it is used in the Flemming triple stain), and of methyl green and acid fuchsin (with which it is combined in the technique of Ehrlich-Biondi-Heidenhain). In the Mallory connective tissue stain it is employed with two other acid dyes, aniline blue and acid fuchsin. A further use is Bensley's "neutral gentian," a combination of orange G with gentian violet for staining the islands of Langerhans.

**Orange GG, GMP.** See orange G.

**Orange MNO, MN.** See metanil yellow.

**Orcein.** A natural dye, closely related to litmus, obtained from certain lichens. It is a weak acid, with a violet color. As a biological stain it seems to have been used chiefly by Unna (p. 425), who employs it for staining connective tissue, plasma fibrils in the epithelium, and for other purposes. Other histologists have used it, however, for certain special procedures, as for coloring elastic tissue.

**Orseillin BB.** An acid dye of the azo series that has been used for staining mycorrhizal fungi.

**Para-fuchsin.** See pararosanilin.

**Para-magenta.** See pararosanilin.

**Pararosanilin.** (Syn. *Basic rubin*, *Para-fuchsin*, *Para-magenta*.) A basic dye of the triphenyl-methane series, the lowest member of the group known as basic fuchsin. See basic fuchsin.

**Pararosolic Acid.** An acid dye, a hydroxyl derivative of triphenyl methane. Generally present in commercial preparations of rosolic acid.

**Paris Blue.** See spirit blue.

**Paris Violet.** See methyl violet.

**Phenolphthalein.** A weak acid dye of the xanthene series, related to the fluorescein dyes (eosin, etc.). It is used primarily as an indicator, having its sensitive range between pH 8.3 and pH 10.0.

**Phenol Red.** An acid dye of the sulphonaphthalein series, one of the longest known of the indicators of this group. Its range (pH 6.8 to 8.4) lies close to neutrality, and the indicator has proved valuable for

some time in urine analysis. Under the microscope it also serves as an indicator of reaction when employed as a vital stain.

**Phenylene Blue.** See naphthol blue.

**Phenylene Brown.** See Bismarck brown Y.

**Phloxine.** (Syn. *Erythrosin BB. New pink.*) An acid dye of the xanthene series—dichlor or tetrachlor tetrabrom fluorescein. It is a distinctly red dye with little of the orange tinge noticeable in the lower homologues, eosin and erythrosin. It proves a good counterstain in contrast to certain blue dyes of the thiazin series. It works better than eosin in the "eosin-methylene-blue" stain of Mallory for sections of tissue. Under certain conditions it is a very good bacterial stain. Chamberlain<sup>11</sup> (p. 229) finds that it gives much better results than magdala red in staining algae; and concludes that the "magdala red" which he used before the war must have contained phloxine or some closely related dye.

**Phosphine.** (Syn. *Leather yellow. Xanthene.*) An acridine dye which has been employed as a microchemical agent for nucleoproteids.

**Picric Acid.** A strongly acid dye of the nitro series, yellow in color. One of the most common uses of picric acid is in contrast to acid fuchsin in the Van Gieson connective tissue stain. It is also employed as a general cytoplasmic stain in contrast to various basic dyes. It has further application as a fixative.

**Pinacyanol.** (Syn. *Sensitol red.*) A quinoline dye that has been employed in staining frozen sections. Its best known use, however, is in photography.

**Ponceau B.** See Biebrich scarlet, water sol.

**Ponceau 3B.** See Sudan IV.

**Ponceau 2R.** (Syn. *Ponceau R, RG, G, 4R, 2RE, NR, J, FR, GR. Scarlet R. Xylidine ponceau 3RS. Lake ponceau. Brilliant ponceau. New ponceau 4R. Rainbow scarlet.*) An acid azo dye, having occasional use as a counterstain in histology, especially in the Masson trichrome technique.

**Purpurin.** (Syn. *Alizarin No. 6. Alizarin purpurin.*) An acid dye of the oxyquinone group, closely related to alizarin. It is a little darker in shade than alizarin. It has been used as a nuclear stain for histological material, and for determining the presence of insoluble calcium salts in cell contents.

**Pyoktanin Blue.** See methyl violet.

**Pyoktanin Yellow.** See auramin.

**Pyoktaninum Aurem.** See auramin.

<sup>11</sup> Chamberlain, C. J. *Methods in Plant Histology*. Univ. of Chic. Press, Ed. 5, 1932, p. 110.

**Pyronin B and Y.** Two very closely related dyes of the xanthene series. Pyronin Y is tetra-methyl diamino xanthene, while pyronin B is the corresponding tetra-ethyl compound. Pyronin Y, under its German designation, pyronin G, was apparently the dye used before the war; but pyronin B seems to be a satisfactory substitute in histological work. Pyronin Y is needed for special procedures only. It is most used in combination with methyl green (the Pappenheim stain) for staining various basophile elements such as mast cells and for staining the gonococcus in smears of pus. In the formula for the Pappenheim stain allowance must be made for the fact that the American pyronins are more concentrated than those used before the war; and a smaller amount in proportion to methyl green should be employed.

**Pyrosin B.** See erythrosin, bluish.

**Pyrosin J.** See erythrosin, yellowish.

**Rainbow Scarlet.** See ponceau 2R.

**Red Violet.** See Hofmann violet.

**Resorcin Blue.** (Syn. *Fluorescent blue. Iris blue.*) A brominated basic dye of the oxazin series. It is often called *lacmoid*, although that name more properly belongs to the unbrominated compound. It has been employed as a microchemical reagent for the detection of callose.

**Rhodamine B.** (Syn. *Rhodamine O. Brilliant pink.*) A weakly basic dye of the xanthene series. It has been used in combination with osmic acid as a solution for simultaneous fixing and staining. It has also been employed for histological work in contrast to methylene blue or methyl green; and as a component of certain compound stains.

**Rhodamine O.** See rhodamine B.

**Romanovsky Stain.** The first compound of eosin and methylene blue proposed for staining blood. It was merely a mixture of the two dyes without effort to precipitate and remove the compound dye formed; and yet its author realized that some new dye was formed in the solution. The methylene blue solution was not intentionally polychromed, although Romanovsky discovered that old solutions gave best results.

**Rosanilin.** A basic dye of the triphenyl methane group, differing from pararosanilin in that a methyl group is attached to one of the benzene rings of the latter. It is a member of the group of compounds known as basic fuchsin. See basic fuchsin.

**Rosazine.** See azocarmine G.

**Rose Bengal.** An acid dye of the xanthene series—dichlor or tetrachlor tetra-iodo fluorescein. It is even more distinctly red than phloxine, to which it is closely related; and often shows a slight violet cast in staining. As a result it is not such a good counterstain to the blue dyes



as the lower members of the group. It has been employed as a bacterial stain, especially for staining bacteria in soil suspensions.

**Rosinduline GXF.** See azocarmine G.

**Rosolic Acid.** (Syn. *Aurin.*) An acid dye, a hydroxyl derivative of triphenyl methane. Its chief use is as an indicator.

**Rubin.** See basic fuchsin.

**Safranin O.** A basic dye of the azin group. There are various safranins but the grade desired by biologists seems to be a certain mixture of dimethyl and trimethyl phenosafranins, having a definite shade of red not easily recognized by eye but characterized by having a light absorption curve with its maximum at or near  $515\mu$ . It is one of the most important nuclear and chromatin stains known. It is especially valuable to the plant histologist for bringing out lignified and cutinized tissues, and as a protein stain. The cytologist makes use of it in the Benda technique to stain chromatin in combination with light green as a contrast stain; and even more widely in the Flemming triple stain, in which it is employed to bring out the chromatin, with gentian violet and orange G for contrast. The bacteriologist uses it occasionally, especially as a counterstain in the Gram technique.

**Safrosin.** See eosin, bluish.

**Scarlet B or EC.** See Biebrich scarlet, water sol.

**Scarlet G or B.** See Sudan III.

**Scarlet J, JJ or V.** See eosin, bluish.

**Scarlet R.** See ponceau 2R.

**Scarlet Red.** See Sudan IV.

**Scharlach R.** See Sudan IV.

**Sensitol Red.** See pinacyanol.

**Silver Gray.** See nigrosin, water sol.

**Solid Green.** See malachite green.

**Soluble Blue 3M or 2R.** See aniline blue, water sol.

**Soluble Yellow OL.** See metanil yellow.

**Spirit Blue.** (Syn. *Gentian blue. Aniline blue, alcohol sol. Night blue. Lyons blue. Paris blue.*) A basic dye of the triphenyl methane group (from which aniline blue, water soluble, is derived by sulphonation). It is a mixture of diphenyl and triphenyl rosanilin, and hence varies in shade according to the proportion of each present. It is seldom used in microscopic work; but has been employed in contrast to carmine in staining embryonic tissues. It brings out growing nerve fibers well.

**Steel Gray.** See nigrosin, water sol.

**Sudan II.** See oil red O.

**Sudan III.** (Syn. *Sudan G. Tony red. Scarlet G or B. Fettponceau G. Oil red. Cerasin red.*) A weakly acid, fat-soluble dye of the azo group,

which colors fat an orange color. It is the first of the oil-soluble dyes to be employed by histologists as a stain for fat in sections. It is still used, partly for staining sections and partly for staining fat in masses of tissue for gross examination; but to a large extent it is now replaced by Sudan IV.

**Sudan IV.** (Syn. *Scarlet red*. *Scharlach R*. *Oil red IV*. *Fettponceau*. *Ponceau 3B*.) A weakly acid, oil-soluble dye of the azo group, a dimethyl derivative of Sudan III. On account of the methyl substitution, it is of a scarlet rather than an orange color and accordingly stands out more vividly in a microscopic preparation. It is used as a fat stain in histology.

**Sudan AX.** See oil red O.

**Sudan G.** See Sudan III.

**Sudan Red.** See magdala red.

**Sudan R.** (Syn. *Brilliant fat scarlet B*. *Oil vermillion*.) A fat-staining dye of the azo series, which has been employed as an aid in reading tubes in the Kahn reaction for diagnosing syphilis.

**Sudan X.** See oil red O.

**Sultan 4B.** See benzopurpurin 4B.

**Swiss Blue.** See methylene blue.

**Tetrachrome Stain** (MacNeal). A mixture of methylene blue, methylene azure, methylene violet, and eosin, used for staining blood films. Recently MacNeal has specified azure A for methylene azure (see azure A). As these dyes are mixed in dry form, no reaction takes place between them. The mixture is dissolved in methyl alcohol, and no immediate reaction takes place. A compound dye is slowly formed, however, with the result that a troublesome precipitate may occur. Accordingly the best way to dissolve the stain is to allow the solution to stand for about twenty-four hours at approximately 50°C. so that the interaction between the dyes will take place as rapidly as possible; and then the precipitate can be removed by filtering. Such a solution keeps some time; but it is not as permanent as Wright's stain. When fairly fresh, it gives as good results as the latter; in fact the blood pictures obtained by the two stains are very similar. The chief advantage of the tetrachrome stain is that it can be prepared from the constituent dyes with a little more precision than Wright's stain.

**Thionin.** (Syn. *Lauth's violet*.) A basic dye of the thiazin series, differing from methylene blue and the azures in not being methylated. Thionin is no longer used as a textile dye; and until recently it was difficult to obtain the correct product when ordering. That difficulty no longer exists, however. It is a very valuable chromatin and mucin stain, especially prized for certain procedures because of its high degree of

metachromasy. It is a useful vital stain. It is employed in staining frozen sections of fresh animal or human tissue, especially in the study of tumors. It is used for staining very young bacterial colonies in the Frost "little plate" technique for counting bacteria.

**Thymol Blue.** An acid dye of the sulphonephthalein series, employed as an indicator, changing from red to yellow through the H-ion range between 1.2 and 2.8, and from yellow to blue between 8.0 and 9.6. May be used as an indicator of reaction in vital staining.

**Toluidin Blue O.** (Syn. *Methylene blue O.*) A basic dye of the thiazin series, closely related to methylene blue, and even more like azure A. Theoretically it should be a good substitute for the latter; and in actual experience it proves possible to employ it instead of azure A in certain procedures called for by the latter. Further investigation may show that it can be substituted for the latter quite generally; and as it is a recognized textile dye, it will be much more readily available. It can be substituted for thionin in staining frozen sections of tissue. It has been definitely specified in certain staining procedures such as in the panchrome stain of Pappenheim (p. 341) and in the Albert stain for the diphtheria organism.

**Toluylene Red.** See neutral red.

**Tony Red.** See Sudan III.

**Trapaeolin G.** See metanil yellow.

**Tropeolin D.** See methyl orange.

**Tropeolin OOO No. 2.** See orange II.

**Trypan Blue.** (Syn. *Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamine blue 3B. Azidine blue 3B. Niagara blue 3B.*) An acid dye of the azo series, which has been occasionally employed in vital staining.

**Trypan Red.** An acid dye of the azo series, which has been used for vital staining.

**Uranin.** See fluorescein.

**Vesuvium.** See Bismarck brown Y.

**Victoria Blue B.** (Syn. *Fat blue B. Corn blue BN.*) A basic dye of the triphenyl methane series, occasionally employed in tissue staining.

**Victoria Blue R.** (Syn. *New victoria blue B. Corn blue B.*) A basic dye closely related to Victoria blue B; it is not certain whether it has been used in staining.

**Victoria Blue 4R.** (Syn. *Fat blue 4R.*) A basic triphenyl methane which has been employed in staining spirochetes in blood.

**Victoria Rubin.** See amaranth.

**Violamine 3B.** See fast acid blue R.

**Violet C, G or 7B.** See crystal violet.

**Violet R, RR, or 4RN.** See Hofmann violet.

**Vital Red.** (Syn. *Acid Congo red. Brilliant Congo red R. Brilliant Congo R. Azidine scarlet R. Brilliant dianil red R.*) An acid dye of the azo series. It is quite an important vital stain.

**Water Blue.** See aniline blue, water sol.

**Water Soluble Eosin.** See eosin Y.

**Wool Orange 2G.** See orange G.

**Wright Stain.** The compound of methylene blue and eosin most commonly employed for staining blood smears. In preparing Wright stain, the methylene blue is heated in flowing steam for an hour, by which treatment it is partly oxidized into methylene violet and the azures—i.e. “polychromed.” As the dyes produced in this oxidation process are all basic, they are capable of entering into chemical combination with eosin. These compound dyes are insoluble in water and are therefore precipitated. For use, they are dissolved in methyl alcohol; and after the alcoholic solution has stood a short time on the blood film it is diluted with water or with a solution of buffer salts. In this mixture of alcoholic and aqueous solutions there are likely to be present the original dyes, eosin and methylene blue, also the oxidation products of the latter, and the eosinates of the various basic dyes in the polychrome methylene blue. As a result, a great variety of staining effects is produced. Wright stain may be produced in the laboratory from methylene blue and eosin, or it may be purchased ready made. The prepared Wright stain now obtainable on the market is very reliable, and is so inexpensive that its preparation by the biologist himself is not necessary, unless it is to be used in large quantities.

**Xanthene.** See phosphine.

**Xylidine Ponceau 3RS.** See Ponceau 2R.

**Yellow M.** See metanil yellow.

**Yellow WR.** See brilliant yellow S.

### Aniline Stains: General Considerations

Aniline dyes may be treated very much as a class and general statements may be made which will therefore be applicable to all of them. They offer a great range of choice in both color and action and now constitute a very valuable resource in microtechnique.

(a) *Solvents.* Most of the anilines are soluble in both water and alcohol. If alcohol is used it may be in strengths of 30 per cent, 50 per cent, 70 per cent or 95 per cent. There are some general advantages in a solution made in 50 per cent alcohol, but on the other hand, especially for counterstaining, it is of advantage to use 95 per cent or even absolute alcohol. In addition to plain aqueous solutions there are those

made in aniline water, which has the advantage of greater solubility and also of some mordanting action. Finally, it is often convenient to dissolve the aniline color in clove oil so that staining and clearing may be accomplished at the same time.

(b) *Strength of Solutions.* The formulas call for different percentages of aniline dyes, but for most purposes it is very convenient to employ saturated solutions. Since generally this approximates the 1 per cent to 2 per cent strength which is called for, it is perhaps the easiest manner of handling the dye.

(c) *Method of Application.* The anilines, like other dyes, are employed as both progressive and regressive stains, but, because of their solubility and the absence of mordants in most cases, they are almost perforce regressive stains. Differentiation may be accomplished by the use of the solvent which was employed in making up the dye, i.e., either water or alcohol. In case of refractory dyes where plain alcohol or water do not work rapidly enough, the addition of 0.1 per cent of hydrochloric acid will facilitate extraction of the color. Where it is desired to introduce two colors into the preparation it is sometimes possible to discharge the first color from portions of the cell or tissue by the use of the second color. Commonly the basic nuclear stain is displaced by the plasma stain. Thus, if gentian violet is used as a chromatic stain it may be differentiated by the action of orange G.

Clearing is accomplished by the use of any of the ordinary reagents like xylol, but for many purposes where additional differentiation of a precise sort is required, a final step may be taken by the use of clove oil, which clears and differentiates at the same time.

(d) *Relation of Stain to the Fixative.* The fixative has, in many cases, an outstanding influence upon the operation of the dye. In general, aniline colors are most effective after fixatives containing chromic and osmic acid. The picro-formol-acetic mixtures are less effective in preparation for aniline dyes than are the osmic or chromic mixtures. In case it is desired to have the advantages of the picro-formol-acetic mixtures and at the same time utilize aniline colors, the end may be attained by adding a small quantity of Flemming's fluid to them. Sometimes it is even possible to mordant sections fixed in the P.F.A. mixtures by treating them with Flemming's fluid before staining.

(e) *Mordanting.* In general the aniline dyes do not require mordants, but for some purposes it is advantageous to attach the dye more strongly to the tissue than is possible by direct action and in this event a mordant, such as aniline water or a solution of potassium permanganate, is advantageous. The mordant is applied as in the cases of carmine and hematoxylin. If potassium permanganate is used, a

1 per cent aqueous solution, acting for five or ten minutes, is usually satisfactory. Iodine may also be used as a mordant. Instead of applying the mordant before the stain, it has been recommended that in the case of iodine it be allowed to operate after the stain is in the tissue.

2. **Inorganic.** Inorganic staining agents are mostly salts of gold and silver which under the action of light are precipitated as metals in the presence of certain substances, like intracellular cement, and within nerve and neuroglia cells. Such effects are commonly spoken of as impregnations and differ materially from the ordinary staining reaction, although they grade into it under some conditions. Since they are used to demonstrate tissue and cellular constituents they may be properly considered along with ordinary stains.

## II. Composition of Staining Agents

1. **Physical Composition.** In their physical state staining agents may be either *simple* or *compound*, that is, they may consist of a single constituent or of more than one element. A consideration of this phase of the subject is of value only in the way of orientation.

2. **Chemical Composition.** In chemical constitution stains may be either acids, bases or salts, or they may be of complicated organic structure. In general they do not exhibit any pronounced acid or basic properties. Sometimes the salts are designated as basic or acid dyes, depending upon whether the radical upon which their staining power depends occupies the position in a compound of a base or an acid. (For discussion of these topics in detail see the sections on the different dyes pp. 575, 603.)

## B. NATURE OF STAINING COMBINATION

### I. Physical Nature

After the stain has been dissolved in a medium there are certain physical characteristics with regard to it which have important bearings upon its action.

1. **Solvent Aqueous or Alcoholic.** The solvent, whether water or alcohol, while it does not alter the characteristic staining reaction of the dye, does have a material effect upon the rapidity and degree of its action. Accordingly a solvent should be chosen which comports most favorably with the particular staining reaction desired. In most cases an aqueous stain is preferable, but sometimes it is quite advantageous, especially in counterstaining, to make the application of the dye late in the process, just before mounting. In this case, of course, an alcoholic

solution should be made, or even one in clove oil when it can be used for clearing.

2. **Concentration, Strong or Weak.** The concentration of the dye in the medium also influences the character of the stain. As a general rule, in progressive staining, a weak solution of the dye, allowed to act over a longer period, gives a more precise and selective effect than when a concentrated solution, acting more rapidly, is used. On the other hand, when it is difficult to secure a reaction between dye and tissue, use a concentrated solution. The solvent effect of the dehydrating agent or clearing agents then produces sufficient differentiation. In very resistant cases a solution of aniline dye in aniline water is more effective than is a plain aqueous solution.

3. **Temperature, Low or High.** The temperature of the solution when staining takes place also influences the nature of the stain. Particularly in the case of blood smears, the temperature of the reaction is important (see Blood, p. 330). Commonly, however, the staining operation takes place best at room temperatures and unless there are particular reasons for doing otherwise, this should be the practice.

4. **Composition, Simple or Multiple.** Staining solutions in their compositions may be either simple or multiple, that is, they may consist of a solution of one dye, or there may be two or more of these. By such combinations it is possible to get contrasting colors combined with different portions of the tissue or cell at one operation. These special combinations will be considered particularly under different headings (see Blood, p. 325). Some of these combinations are more than mere mixtures, being in effect chemical unions between a staining base and a staining acid of different colors. Polychromatic or metachromatic effects of beautiful clearness result from the use of such stains.

## II. Chemical Nature

1. **Acid, Basic or Neutral.** The chemical composition of combinations, quite aside from the chemical reaction of the active dye, may be either acid, basic or neutral. Sometimes, as in the aceto-carmine mixture of Schneider, the acid is present in strong concentration and so acts as a fixative. Similarly, methyl green combined with acetic acid forms a strongly acid combination which fixes and stains simultaneously. Various combinations are rendered acid in order to secure greater vigor and precision in their effects. On the other hand, only a few staining combinations are basic. The most outstanding examples of such a stain are Beal's borax carmine and ammonia carmine. These alkaline stains were in the early days much used, but at present are

only infrequently employed. Most stains are now made up in neutral media.

### C. APPLICATION OF STAINS

#### I. Intra Vitam

Intra vitam stains are those which are applied to the tissue in its living condition. Such stains are called "vital" stains or "supravital" stains. For discussion of intra-vitam stains see chapter on fresh material (pp. 110, 117).

#### II. Post Mortem

Post-mortem application of stains, or their use on tissues after they have been killed and fixed, is the common practice; therefore most of the discussion in this chapter will be on this subject. The practical application of stains in this way is influenced by (a) the character of the material and (b) certain relations to the stains.

1. **The Character of the Material to Be Stained.** This may be indicated under the categories of whole organisms, whole organs, tissues, or cells.

*a. Whole Organism, Entire Organ, Tissues and Cells.* If the organism is small it is convenient, and in every way desirable, to stain it entire. This obviously has many advantages because the parts are all seen in their normal relations and, by the selection of proper staining agents, may be well differentiated from each other. In general, the best stains to use for such a purpose are the carmine mixtures, either alone or combined with picric acid. Sometimes it is desirable similarly to study single organs entire and they may be treated in the same manner. In the majority of cases, however, the differential staining reaction takes place upon tissues or cells, that is, the stains are classified as either histological or cytological. It is possible to differentiate well between tissues in a very striking and characteristic manner as is done, for instance, by the use of Mallory's connective tissue stains, or the various neurological stains. Finally the parts of cells may be distinguished by specific staining reactions. Perhaps the most general and characteristic of such differential effects is that obtained by the use of nuclear stains which pick out these cellular elements by their specific staining reactions.

2. **State of Material to Be Stained.** *a. In Toto.* Staining in toto was formerly much practiced, but latterly has fallen into considerable disuse. This method, however, has certain advantages which, if carefully utilized, make it one of great convenience. It is perhaps most



commonly used in embryological work where the whole organism is stained before it is sectioned. The value of this method depends largely upon the character of the staining agent and the choice here is rather strictly limited. Neither the aniline stains nor hematoxylin lend themselves well to such use. There remain, therefore, practically only the carmine combinations. Perhaps the best of these, for most purposes, is not a combination including carmine itself but rather the alum-cochineal stain. This gives a precise effect and also has the advantage of not overstaining. Properly controlled, a very exact differentiation, not only between tissues but also between cellular elements, can be secured (alum-cochineal).

*b. Sections.* Sections are usually selected as best for application of stains. If the *paraffin* method has been used, the sections are affixed to the glass slip before staining, whereas most *collodion* sections are stained before they are mounted. This is not obligatory, however, and if serial sections are prepared by the collodion method it is almost necessary to affix them to the glass before staining is attempted. For description of method of staining sections see Part 1.

*c. Dissociated.* Dissociated materials, as a rule, stain with difficulty because the dilution of the fixing agent required for securing the macerating effect results in an indifferent staining reaction. It is therefore usually desirable to use a vigorous stain like Delafield's hematoxylin, although by proper treatment picro-carmine may be made to render an acceptable result. After the fixative is washed out the stain is added to the fluid in which the cells are floating and allowed to act until the desired effect is produced. It is sometimes convenient to continue this treatment through all the successive steps involved in mounting in balsam. When this is done, a drop of balsam containing the cells, when placed under a cover glass, will give a complete preparation.

*d. Stretched.* Stretched material is essentially the same as sections except for the fact that there are no cut surfaces through which the stain can penetrate. The methods employed for sections can therefore be applied directly.

**3. Relation of Material to Stain.** The relation of the material to the stain may be influenced by the character of its action and by the time and degree of action.

*a. In Action.* In action stains may be substantive, adjective, or impregnation stains. A *substantive stain* is one which acts immediately and directly upon the tissue without the intervention of any other substance. An *adjective stain* is one in which the tissue is first treated with some agent which in turn attaches the stain to the tissue. *Im-*

*pregnations* are deposits of sensitive metallic substances in tissues under the action of light.

*b. In Time.* Relation of material to stains in time of action. The character of staining depends upon the time of its action on the tissue. A selective action upon any one tissue or cell constituent may be obtained by watching the degree of stain. By observing sections under the microscope, therefore, the desired differentiation is secured directly.

*Progressive Staining.* Such a practice is known as progressive staining. For many purposes the precision thus reached is satisfactory, but it is obvious from the nature of the process that there must be a gradation of effect which would prevent the sharpest differentiation.

*Regressive staining*, therefore, is resorted to when the most extreme sharpness of differentiation is desired. By this method the whole tissue is completely stained and then, by removing the excess dye from the parts desired unstained, by careful control under the microscope, an extreme precision of differentiation may be attained. Such a method may be applied to all classes of stains. It is the one which is utilized in the classical method of Heidenhain in his iron alum-hematoxylin combination. It is almost obligatory in the case of aniline stains because, in the process of dehydration, and to some extent in that of clearing where essential oils are used, a differential solution of the stain is necessarily accomplished.

*c. In Degree.* In the degree of their action upon tissues, stains may be classified as general or selective.

*A general stain* is one which attacks all parts of the tissue with approximately equal vigor and thus produces no marked differentiation. Sometimes by regressive methods a general stain may be made to exhibit more selective action. Such general stains are more commonly found among the anilines than in natural stains. Much of selective action is due to previous treatment of the material and to method of applying the stain rather than to inherent differences in dyes.

*Selective stains* may differentiate between classes of tissues or between parts of cells. Certain stains attack only particular tissues, as orcein does elastic tissues, or Sudan III fat, etc.

*Plasma and Nuclear.* In cytological work there is a series of stains which have specific effects. Although we have no exact chemical tests, cytological stains generally fall under the headings of nuclear or cytoplasmic. Nuclear stains are primarily those which affect chromatin, although there are certain ones which, in a measure, have a differential action upon the so-called achromatic structures. The natural stains are primarily nuclear stains and this is true also of basic aniline dyes. Of stains differentiating cytosomic structures there is correspondingly a

whole series. Many of the aniline dyes may be utilized for differentiating the general cytoplasm as opposed to the nucleus. There are, however, certain more specific agents which are used for distinguishing mitochondria and the Golgi apparatus. This subject will be found discussed more in detail in the section dealing with these topics (pp. 265, 274). Certain aniline dyes are only slightly toxic and have specific selective action on constituents of living cells (p. 117).

## D. FORMULAS

### I. Aniline Dyes

Single aniline dyes are simple solutions in either water, alcohol or clove oil. For stronger action, aniline water (distilled water saturated with aniline oil) is used as the solvent. The strength of the solution, except for the vigor of the action, is not important, since differentiation always attends dehydration. There are, however, a number of combination stains which are of great value and which sometimes are of exact composition. Because of variations in materials to be stained and uncertainty in the chemical constitution of aniline dyes it is difficult to give exact directions regarding strength of solutions and relative time of action where more than one dye is used. The only practical plan when trying new dyes on new material is to test out the solutions and their operation until the desired result is obtained. There are a very large number of multiple aniline stains, only a few of which have general application. Among these are the following:

*Flemming's Tri-color Stain.* Although according to the dictum of Lee "never popular, this clumsy and uncertain process is now little used," this method is one of the most useful and popular of the combination aniline stains. It admirably supplements the Heidenhain iron hematoxylin method and has some advantages over it in the contrasting colors shown in prophases of mitosis and in the transparency of the metaphase chromosomes. As suggested elsewhere it should form a routine alternative to the use of the hematoxylin stain in cytological work. There are many modifications of the strengths of solutions and methods of application, but these are of little consequence since they represent adaptations to particular cases. With the proper choice of aniline dyes to begin with, the details of strength of solution and time of application can easily be adjusted to each case. Due regard should be had to the character of the illuminant when the safranin and gentian violet are chosen (p. 616). The following solutions will be found satisfactory in the majority of cases:

- (1) Saturated alcoholic solution of safranin, 95 per cent. . . . 1 part  
Aniline water . . . . . 1 part
- (2) Gentian violet, saturated aqueous solution
- (3) Orange G, saturated aqueous solution

Sections fixed in either Flemming or Hermann, or after fixation in other fluids mordanted in osmic acid solution, are stained for two to twenty-four hours in the safranin. They are then rinsed in water, and transferred to the gentian violet solution for an appropriate time, for example, thirty minutes to two hours. They are then removed from the staining jar and on them is dropped the orange G solution which is allowed to act thirty to sixty seconds. The object generally aimed at is to secure a stain which shows the chromosomes in metaphase stained clearly with the safranin, while the prophase chromatin takes the gentian violet. The purpose of the orange G is not so much to add a third color, but rather to differentiate between the red and the violet dyes.

Differentiation is accomplished by dropping on the slide 95 per cent alcohol until clouds of color cease to come from the sections. After this treatment, clove oil is dropped on the sections, and final differentiation accomplished by observation under the microscope. The action of the clove oil is slow, so that a very exact differentiation may be secured. Some workers prefer to differentiate the safranin before adding the gentian violet and there are various other modifications of the process, but the end-result sought in each case is to obtain in different portions of the cell accurate segregations of the two principal colors in the stain.

The action of this stain is so precise under proper manipulation that in one strand of chromatin the diffused portion will take the violet, while the condensed section will be stained with the safranin.

*Wright's stain* (Blood, p. 326).

*Leischmann's stain* (Blood, p. 326).

*Romanowsky's stain* (Blood, p. 326).

*Van Gieson's stain* (Section on Connective tissue, p. 405).

*Mallory's aniline blue connective tissue stain* (Section on Connective tissue, p. 469).

*Mallory's acid fuchsin* (Section on Connective tissue, p. 404).

*Feulgen's reaction* (p. 629).

## II. Carmine and Cochineal

### 1. Carmine Stains, Alkaline and Neutral.

a. *Ammonia-carmine*. Ranvier introduced a stain prepared by dissolving carmine in water with a slight excess of ammonia. Upon evaporation a substance is produced which, when subsequently dissolved in water, makes an excellent stain.

b. *Picro-carmine*.<sup>10</sup> In a saturated solution of picric acid add carmine, dissolved in ammonia, until no more is taken up. Evaporate to  $\frac{1}{3}$  of the original volume and filter off the precipitate which forms when the liquid cools. Evap-

<sup>10</sup> Ranvier, *Traité*, p. 100.

orate the filtrate to dryness, which will give a crystalline powder somewhat the color of red ochre. For use prepare a 1 per cent solution of this.

*c. Alcoholic Carmine Stains.* Borax-carmine. In a 4 per cent solution of borax dissolve 2 per cent of carmine by boiling for half an hour. Allow to stand for two or three days and dilute with about an equal volume of 70 per cent alcohol and then filter. After staining, bring the material into 70 per cent alcohol acidulated with 5 drops of hydrochloric acid in each 100 c.c. of alcohol. When the material has become clear and transparent wash out the acid in alcohol. This is very valuable for staining in bulk.

## 2. Carmine Stains, Acid.

*a. Alum-carmine.*<sup>11a</sup> In 5 per cent aqueous solution of ammonia alum boil for ten to twenty minutes 1 per cent of powdered carmine. Cool and filter. This is very easy to use since it will not overstain.

*b. Carmalum, Mayer's.*<sup>12</sup> In a 5 per cent aqueous solution of ammonia alum dissolve 1/2 per cent of carminic acid, using heat if necessary. Filter and add 0.1 per cent of salicylic acid. This is very valuable for in toto staining. Wash out the excess of stain in distilled water which will leave the cytoplasm colored. If a purely nuclear stain is desired use an alum solution for washing.

*c. Alum-carmine and Picric Acid.*<sup>13</sup> Mix 10 volumes of alum carmine with 1 volume of saturated picric acid solution. With this a double stain may be secured.

*d. Schneider's Aceto-carmine.* Heat 45 per cent strength acetic acid to the boiling point and dissolve in it as much carmine as it will take up. Filter and preserve the filtrate. This is a very valuable stain of somewhat limited application. Fresh material may be fixed and stained at the same time but the specimen cannot well be preserved afterward. It stains the nucleus almost alone.

A modification of this stain has been used recently by Belling. His formula is as follows:

Mix equal volumes of glacial acetic acid and water. Add to this powdered carmine in excess. Boil, cool and filter. To the solution add a few drops of ferric hydrate dissolved in 50 per cent acetic acid. This acts as a mordant and may be varied in proportion to suit the requirements of different objects. The amount of iron affects the color of the stain. The greater the proportion of the iron, the darker and bluer the stain. This is particularly valuable in making rapid studies of chromosome numbers in smears. The preparation may be examined directly in the stain. Such preparations are fugitive but may be preserved for a considerable time by sealing the edges of the cover glasses with vaseline or some other substance.

## 3. Alum-cochineal.

Powdered cochineal .....	12 gm.
Potassium alum .....	12 gm.
Water .....	160 c.c.

<sup>11a</sup> Grenacher, *Arch. f. mikr. Anat.*, vol. 16: 1879.

<sup>12</sup> Mayer, *Mitth. Zool. Stat. Neapel.*, vol. 10: 1892.

<sup>13</sup> Legal, *Morph. Jahrb.*, vol. 8.

Dissolve the alum in the water and boil the cochineal in the mixture for twenty minutes. Decant the clear supernatant liquid, add water to the cochineal, and boil again. Decant and add to the first liquid, filter and evaporate to 160 c.c. Add a crystal of thymol to prevent the growth of molds. This is largely used for staining in toto. For this purpose place the piece of tissue in the stain for twenty-four to forty-eight hours. Wash in water twenty to sixty minutes to remove the alum, but in order to avoid the extraction of the stain run up through the grades of alcohol to 70.

### III. Hematoxylin and Hematein

a. *Delafield's hematoxylin*.<sup>14</sup> Prepare 400 c.c. of saturated solution of ammonia-alum. To this add 4 gm. of hematoxylin crystals dissolved in 25 c.c. of 95 per cent alcohol. Expose to the light and air for three or four days. Add 10 c.c. of glycerin and 100 c.c. of methyl alcohol and filter. By being exposed to the air it will slowly ripen, but this process may be rapidly completed by adding a small quantity of hydrogen peroxide. This solution keeps indefinitely. In the undiluted condition it is a very powerful and somewhat general stain, but in dilute solution it acts more slowly and specifically. This is one of the most common stains used in clinical studies and is a valuable laboratory reagent. Very often it is used with eosin as a counterstain, giving a strong contrast between the nucleus stained blue with hematoxylin and the cytoplasm pink with eosin.

b. *Mayer's hemalum*.<sup>15</sup> Dissolve 1 gm. of hematoxylin in 1 liter of water. To the solution add 0.2 gm. of iodate of sodium and 50 gm. of alum. This is an improved formula of Mayer's original hemalum. It acts very much like Delafield's but the solution does not keep so well.

c. *Mayer's acid-hemalum*. This is a stain made by adding 2 per cent of glacial acetic acid to the hemalum stain.

d. *Von Möllendorff's iron hematoxylin*.

e. *Mallory's phosphotungstic acid hematoxylin* (p. 427).

f. *Mallory's phosphomolybdic acid hematoxylin* (p. 290).

g. *Mallory's ferric chloride hematoxylin*. Mordant in 10 per cent ferric chloride for five minutes; rinse quickly and stain in 1 per cent hematoxylin, five minutes, and differentiate in 0.25 per cent ferric chloride (See Heidenhain's stain below).

h. *Verhoeff's stain* (p. 414).

i. *Heidenhain's Iron Alum Hematoxylin*.<sup>16</sup> This is used in two solutions: (1) a mordanting solution consisting of a 4 per cent aqueous solution of ferric alum (ammono-ferric sulphate). In preparing this be careful to select crystals that are all a pure violet color and not oxidized. (2) A 1/2 per cent aqueous solution of hematoxylin—best prepared by first dissolving the hematoxylin crystals in a small quantity of 95 per cent alcohol. In use the sections are first placed in the mordanting solution for a period of time running from one-half hour to twenty-

<sup>14</sup> Delafield, *Ztschr. f. wissenschaft. Mikr.*, vol. 2: 1885.

<sup>15</sup> Mayer, *Ztschr. f. wissenschaft. Mikr.*, vol. 20: 1923.

<sup>16</sup> Festschr. f. Kolliker, vol. 3: 1892.

four hours. From this they are brought into water where the excess of the alum solution is removed. This is important, since if the alum is carried over into the hematoxylin solution it injuriously affects it. After washing, the sections are brought into the hematoxylin solution where they are left for a period of time equal to that of mordanting. At the end of the operation they will be entirely black throughout without any differentiation. It is then necessary to remove the excess stain, which is done by use of the mordanting solution. If this works too rapidly it may be diluted to 2 per cent strength. This operation must be watched carefully under the microscope and at its conclusion the sections are thoroughly washed in water (fifteen to sixty minutes). This is important. The character of the stain seems to be improved by dipping the sections into water at intervals during the process of differentiation. The easiest way to accomplish this is to examine the sections in the water rather than in the alum solution. This is perhaps the best cytological stain which we possess, since it is very precise and exact in its operation, and, because of the method of differentiation, is very flexible. The length of mordanting and staining has some effect upon the final result. Longer treatments give a greater range of action. The color is somewhat affected by the length of treatment also. In this respect, however, the most pronounced effect is due to the age of the solutions, particularly of hematoxylin, fresh stains being bright blue, while older stains range towards black and finally to a rusty green color.

*j. Conklin's Modification of Delafield.* A valuable stain for eggs, where the presence of yolk masks nuclear structures, because of its affinity for hematoxylin.

Take 10 c.c. of Delafield's hematoxylin and 40 c.c. of water, and add 10 drops of Kleinenberg's picro-sulphuric acid mixture. Stain sections for five to ten minutes and wash in alcohol. The presence of the acids in the mixture prevents the yolk from staining and allows the hematoxylin to act upon the chromatin alone.

### *Hematein Stains*

#### *a. Mayer's muchematein.*

Hematein .....	0.2 gm.
Aluminum chloride .....	0.1 gm.
Glycerin .....	40 c.c.
Water .....	60 c.c.

Dissolve the hematein in the glycerin and add the other substances. (For the use of much hematein, see p. 416.)

*b. Orcein* is a dye obtained from certain lichens such as *Lecanora parella*. It is of a violet color and gives metachromatic effects—nuclei blue and cytoplasm pink.

Orcein .....	2 gm.
Glacial acetic acid .....	2 gm.
Distilled water .....	100 c.c.

Wash in water, dehydrate, clear and mount in cedar wood oil. (For the use of orcein in staining elastic tissue, see p. 425.)

## CHAPTER XI

### MISCELLANEOUS

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APPARATUS 615. Handling small objects 615. Illuminants in relation to stain 616. Filtering solutions 616. Cements 617. MATERIALS 617. Mounting media 617. Reagents 618. Preparation of reagents 620. METHODS 621. Decalcification 621. Orientation methods 621. Bleaching methods 623. Huber's water-on-knife method for cutting paraffin sections 623. GENERAL 625. Alligation 625. Thickness of sections 625. Handling cover glass preparations 626. Preservation of specimens 626. Tinting light colored specimens for identification 627. Air bubbles 627. Adipose tissue 628. Artifacts 628. Feulgen's stain 629. Rapid Method for Staining Exposed Surfaces or Sections of Spinal Cord and Brain Stem 630. Fused Quartz Rod Method of Illuminating Living Structures 632. Microincineration Method of Demonstrating Mineral Elements in Tissues 643. Centrifuge Microscope 666. Fluorescent Microscopy 671.

#### A. APPARATUS

##### I. Handling Small Objects

Oftentimes it is desirable to prepare one or many very small specimens which are easily lost in handling. One of the best means of overcoming this difficulty is to use round bottomed shell vials or even a specially shaped one employed in centrifuging. The objects are brought to the bottom of the container, either by gravity or by use of the centrifuge, and the supernatant fluids withdrawn and new ones added. This is a very satisfactory arrangement for carrying through quantities of small eggs and other such substances. Another device employed by Boveri was to enclose the objects in a small packet of frog skin which was held together by a pin which also served as a handle to manipulate the bag. It is even possible by this arrangement to section the preparations and the enclosed material at the same time. Other membranes such as amnions may be employed in the same manner. For the special purpose of handling numbers of small eggs Lefevre devised a special form of watch glass with a small trough in the bottom, of a size which would be satisfactory for forming the block of paraffin in which the eggs are sectioned. With this special watch glass the eggs are more easily brought into a



small compass and it has also the advantage that the final step of infiltration and imbedding may be accomplished in the same container. If a single small object is to be handled it introduces greater difficulties and many devices have been brought forward for overcoming them. (For a description of these see Orientation methods, p. 621.)

## II. Illuminants in Relation to Stain

By due consideration of the relation between the color of stain and that of the light employed, greatly improved results can often be obtained. In the discussion of aniline dyes reference has already been made to the desirability of choosing the right contrasting shades of red and violet for the tricolor stain to be used with artificial illumination. If a standard source of illumination is always used it is possible by proper tests to select a series of colors which will work best under these conditions. However, in the event that this has not been done, improvements may often be effected by the action of color filters; those of the Wratten and Wainwright series are readily available and in their cheaper forms offer no difficulties in the way of expense. By the use of a green color screen in connection with a red dye a very strong contrast approaching a black and white result is obtainable. These devices are of value in visual observation, but when it comes to the use of photography for making permanent records they are almost indispensable. By the proper selection of color filters a considerable range of effects may be secured from the same preparation which makes its interpretation very much more complete than does a single photograph. For a full discussion of such optical aids reference should be made to works on photomicrography.

## III. Filtering Solutions

With the great magnification employed in microscopical work the presence of foreign substances is not only displeasing to the eye, but also very inconvenient or even detrimental in observation of the optical images.

While it is difficult to avoid these intrusions entirely, their presence can be greatly reduced by careful filtering of the solutions employed, at frequent intervals. It is a good practice immediately before using a solution to run it through filter paper. Unless this is done as a matter of routine, operators grow careless and may suddenly find that a fungus has been growing in one of the stains and is present in unpleasant profusion in the preparations.

## IV. Cements

For sealing temporary mounts vaseline or paraffin is used. A very convenient way of applying the latter is to take one of the small candles used for cake decorations, light it and allow it to burn until the paraffin melts and then, with the specimen all ready for ringing, the light is blown out and the wick is used as a means of applying the melted paraffin. For permanent mounts it is often of advantage to surround the mounting medium with a cement, although this practice is less in use than was formerly the case, because the modern mounting media are not so subject to change as was Canada balsam. Of the substances used for ringing covers asphaltum is one of the oldest and best. Orange shellac colored with Bismarck brown also makes an excellent ring. Solutions of celluloid in acetone or amyl acetate or butyl alcohol, or some of the trade preparations like Duco afford satisfactory results.

## B. MATERIALS

### I. Mounting Media

1. **Aqueous.** Some preparations are best preserved in aqueous rather than in balsamic mounting media. The commonest of these are Farrant's medium and glycerin jelly.

#### Farrant's medium

Gum arabic .....	30	gm.
Glycerin .....	30	c.c.
Arsenous oxide .....	0.1	gm.
Water .....	30	c.c.

Keep in stoppered bottle with a lump of camphor.

#### Glycerin jelly

Soak a quantity of gelatin in cold water for two hours.

Warm in a water bath until melted.

Add glycerin 1½ parts.

Filter and add a few drops of carbolic acid.

In the use of these it is of course unnecessary as well as undesirable to dehydrate the material. It should be brought either directly into the mounting medium from water or through a mixture of water and glycerin in the case of glycerin jelly. It is very difficult to prepare mounts of this character since, for best results, the slide should have built upon it a ring of cement of sufficient height to hold the requisite amount of mounting medium. After the excess is carefully removed, leaving the glass slip entirely dry, the mount is sealed by ringing it with cement.

2. **Balsamic.** The balsams are used as mounting media and the common ones are Canada balsam and damar. Sometimes colophonium or resin is employed for this purpose. It is important that only the best quality of these balsams be selected, since if there are acids or other impurities present, the stains are injuriously affected. Because it has been very difficult to get Canada balsam of a satisfactory character in recent years, the use of gum damar has much increased. In preparing this mounting medium select only the clearest and purest tears and dissolve these in benzol to form a thin syrupy solution. This is carefully filtered in order to remove all suspended matter and evaporated to a proper consistency. If it is found in mounting specimens that the benzol is too rapid in its evaporation the original solution may be reduced to a very thick syrup and diluted with xylol or toluol. If the mounts are small, and rapid hardening is desired, the solution in benzol is a very desirable one, but for ordinary purposes the xylol mixture seems to be the best. Other media such as chloroform or turpentine may be used as solvents, but the ones previously mentioned seem to be most satisfactory.

In order to prevent the formation of acid in the balsam it is sometimes wise to add a small quantity of some carbonate like calcium carbonate to the bottle of balsam.

## II. Reagents

1. **Acetone** has only limited application in microtechnique. It is an almost universal solvent and because of this fact might have very important applications. On the other hand, its solvent power makes it difficult to utilize. It may be employed as a dehydrating agent in place of alcohol. It may also be used as a fixative but so far has not been highly recommended for this purpose.

2. **Alcohol.** Ethyl or grain alcohol is one of the most used substances in microscopical technique. It serves as a fixative, a solvent and a dehydrating agent. It is in the latter capacity that it finds the greatest employment. Educational institutions may obtain 95 per cent alcohol through the Internal Revenue Department by making application according to the requirements of the Department. Individuals can purchase 95 per cent alcohol best when it has been denatured. In some cases the denaturant injuriously affects the alcohol for technical purposes, but if nothing is added except methyl alcohol it is entirely satisfactory, provided the strength has not thereby been reduced. For most purposes at least 95 per cent alcohol is necessary for proper dehydration, although in case aniline oil is employed for clearing, much lower grades may be used.

Certain grades of methyl alcohol are quite satisfactory for technical use, but if grain alcohol can be obtained it is the best reagent. Absolute alcohol may be secured by removing the remaining water in 95 per cent alcohol by distillation over quicklime. A convenient method of doing this is to shake the alcohol up with lime and allow it to stand for some time. Then a quantity of this alcohol, together with some fresh lime, is placed in a still and by heating with an electric hot plate the alcohol is slowly distilled off leaving the water combined with the lime. Absolute alcohol should be carefully protected from contact with air because it very quickly takes up moisture and becomes reduced in strength. Another method of increasing the strength of alcohol without distillation is to treat 95 per cent alcohol with exsiccated copper sulphate. This takes up water from the alcohol and it is practical to operate by having some of the copper salt at the bottom of a bottle from which the alcohol is poured off as desired. This is less desirable than distillation over lime, since some of the copper remains in solution.

Exsiccated or anhydrous copper sulphate is prepared by driving off the water of crystallization with heat. A convenient way of doing this is to place the crystals of copper sulphate in a crucible which is then heated over a blast flame until the blue color is entirely removed, leaving behind a white substance. This is powdered in a mortar and it is then shaken up with the alcohol.

For some purposes absolute alcohol is not required. The commercial grade of 95 or 96 per cent is sufficiently high to secure dehydration. This is especially true if the intermediate step of a mixture of equal parts of dehydrating and clearing agent is employed.

**3. Acid Alcohol.** Acid alcohol is used for cleaning glass slips and also for decolorizing after the use of certain stains. Either 70 or 95 per cent alcohol may be used, but commonly the former. Various strengths are employed. A 1 per cent solution is particularly useful in decolorizing. The action of this is rather rapid on sections so the process of decolorization should be watched under the microscope.

**4. Alums.** These substances are double salts containing a large amount of water of crystallization. They are used in microscopical technique as mordants and to form lakes with the staining agents. The commonest in use is the aluminum potassium sulphate. When not otherwise indicated this is the form to be employed in making up solutions of hematoxylin. The aluminum ammonium sulphate or ammonia alum is a cheaper substance and may without injurious results be used as a substitute for the potash alum. Ammono-ferric sulphate, or iron alum, is a salt in which the place of the aluminum is occupied by iron. The crystals of this alum, instead of being clear and colorless, like the potash

and ammonia alums, are violet colored. On exposure to the air they oxidize and thereby become unavailable for use in the Heidenhain technique. It is therefore necessary to select, out of the mass of crystals, only those which are perfect in color. This salt is very deliquescent and should be kept from exposure to moisture. In very warm weather it may melt down in its water of crystallization.

5. **Amyl Nitrite.** This substance is used as a dilator of the peripheral capillaries and is valuable preceding injections where it is desired to obtain a complete injection of the small vessels. This effect may be obtained by adding amyl nitrite to ether at the time of anesthetization.

6. **Benzol** ( $C_6H_6$ ) is the lightest of the benzene ring series. It is used as a solvent for balsam and paraffin and may therefore be employed as a clearing agent. Because of its rather high volatility, however, it is not as frequently used for this purpose as is xylol.

7. **Formalin**, otherwise known as formol or formolose, is a 40 per cent aqueous solution of formaldehyde. It is used in various strengths commonly stated in terms of the aqueous solution. For fixation a 10 per cent aqueous solution is commonly used which is prepared by adding 1 part of formalin to 9 parts of water.

### III. Preparation of Reagents

1. **Mayer's albumen** is a mixture of equal parts of fresh egg albumen and glycerin to which has been added some preservative like sodium salicylate or thymol. The proportions recommended by Mayer are 50 c.c. albumen, 50 c.c. glycerin, 1 gm. sodium salicylate. The original directions require the filtration of this mixture, but this is exceedingly difficult and time-consuming. It may be easily avoided by a simple expedient. Place the fluid in a tall cylinder and shake vigorously until a great many air bubbles are included. These will rise to the surface and carry with them the fragments of membrane which it is desired to remove. This upper layer can thus be taken off, leaving behind a clear mixture quite as good as that obtained by filtration.

2. **Paraffin** of various melting points may be purchased, and, as was indicated in the description of the paraffin method (p. 6), the one utilized should be chosen with careful consideration of the density of the object and the temperature of the room in which the sections are cut. New paraffin is apt to crystallize more readily than that which has been used, and it is the practice of some technicians to melt the fresh paraffin and keep it in this condition for a considerable period of time so that it may become of a more waxlike consistency. It is possible to improve the consistency by the addition of castor oil or India rubber in small quantities. The paraffin which is trimmed from the blocks around

specimens is returned to the jar and remelted. After considerable use paraffin becomes contaminated with foreign materials, in part from the specimens, and in part from the air, and it is necessary to filter these out. The best way to accomplish this is to melt the paraffin and pour it into a paper funnel which has had the apex cut off and in which a wad of absorbent cotton has been placed. If this is placed upon a heated radiator the paraffin will remain melted and in passing through the cotton will leave behind the foreign substances. It is very necessary to avoid the presence of hard gritty material in the paraffin which would injure the knife edge.

It is desirable after the paraffin has been melted and filtered to cool it rapidly by immersing the vessel in cold water. If volatile products are present these will be forced towards the center of the mass which will remain spongy. This portion may be cut out before remelting and thus improve the consistency of the paraffin.

#### *Rubber Paraffin*

Johnson's asphalt formula.<sup>1</sup>

Crude India rubber, cut small..... 1 part

Paraffin, melted and tinged an amber color with asphalt... 99 parts

Heat to 100°C., twenty-four to forty-eight hours.

Remove supernatant fluid and cool. Use as ordinary paraffin.

3. **Preserving Fluids.** Tissues may be kept indefinitely in 70 per cent alcohol and this is a standard medium for the purpose. Several years' immersion in this fluid seems to have no bad effect upon delicate cytological details. If the picro-formol-acetic mixtures have been employed for fixation a gradual extraction of the picric acid will occur, which colors the alcohol but which apparently has no harmful effect. Solutions of formalin of 4 per cent also may be employed for preserving purposes. In general this seems to have no marked injurious effect.

## C. METHODS

### I. Decalcification

This is a process of removing the calcareous substances from such tissues as bone and teeth (bone and teeth, pp. 346, 360).

### II. Orientation Methods

In many cases it is necessary to define the plane of future sectioning accurately before inclusion of the specimen in the imbedding medium.

<sup>1</sup> Johnson, *J. Applied Micro.*, 6: 2662, 1903.

It is very difficult after imbedding to establish a plane of sectioning with regard to the particular elements of structure. Therefore, at the time of imbedding the material is so arranged that by placing certain guides in proper relation to the knife, the desired plane can be realized. As was indicated in the description of the paraffin method, this may be accomplished by placing the tissue upon a piece of paper to which it adheres and so arranging it that the paper is parallel to the desired section plane. In other cases threads containing lamp black are laid in the paraffin and after hardening these are drawn through, leaving marks indicating the plane of section desired.

Very small objects cause unusual difficulties in orientation and special means are necessary. One of these is described by Heuser as follows:

A binocular microscope is mounted on a long swinging arm, so that it can be moved over the dishes which hold the specimens. The latter are thus always kept in view while they are being changed from one fluid to another. When they have been carried up to paraffin they are handled with a long slender glass pipette which is wrapped with coils of small resistance wire and heated with an electric current of proper voltage. A small rheostat controls the strength of the current so that the temperature is maintained just at the melting point of the paraffin. The infiltrating dishes are kept at a uniform heat in a shallow box, open in front for manipulating the instruments and closed on the remaining sides with thin wood boards. An automatic electric hot plate provides a bottom for the box. The top is made of glass; above it is mounted a 100 watt electric lamp which supplies some of the heat for liquefying the paraffin and also gives light.

After the object has been thoroughly infiltrated it is imbedded beside a small stained bit of tissue, for example, a slender column cut from an embryonic liver. These orienting pieces of tissue are prepared in advance and imbedded. Then when one is needed the paraffin above it is melted with an electric needle, the working point of which is made of copper, and the specimen deposited at the lower end of the tissue guide. The specimen is finally arranged as desired in relation to the long axis of the orienting guide block. The specimen cannot be conveniently moved about with the same electric needle that is used to melt the paraffin since eddy currents are set up in the fluid, which are apt to carry the specimen far from the desired position. There is the possibility, too, of injuring the specimen by touching it with the hot instrument, although the temperature of it need not be much above the melting point of the paraffin. The specimen rests upon the solidified paraffin which obviates any danger of superheating. A very useful instrument is one made by soldering to the copper point of the electric needle a second point of iron or German silver wire so that the 2 points stand apart at an angle of about 90°. Either the hot copper point or the warm point can be used as occasion demands by revolving the tool, both the tool and the specimen being under constant observation. With these devices specimens as small as mammalian eggs can be placed in any desired position. Very small objects

cannot be seen in the cold paraffin but since its position in relation to the guide block is known it is a simple matter to make the proper adjustments on the microtome. When the sections are cut those including the guide are discarded and only the ones carrying the specimen are mounted so that the slide is unincumbered with any foreign material.

Fry<sup>2</sup> describes methods of handling small objects in the various steps of fixation, washing, etc. For orientation he recommends imbedding in small water color dishes under a dissecting microscope, in such a manner that the bottom of the vessel indicates the plane of sectioning. This paper contains a list of references for other methods of treating minute objects.

### III. Bleaching Methods

Specimens containing a natural pigment or darkened by the action of osmic acid may be bleached, either entire or after sectioning, in hydrogen peroxide, chlorine mixtures, or sulphurous acid.

#### 1. Hydrogen Peroxide Method.

Hydrogen peroxide .....	3 c.c.
Water .....	97 c.c.

Allow the solution to act until desired effect is secured. After a time maceration begins and the material should be removed to alcohol.

#### 2. Mayer's Chlorine Method.

To a few crystals of potassium chlorate in a bottle or test tube add 2 or 3 drops of hydrochloric acid. When chlorine fumes are evolved add 5 to 10 c.c. of 70 per cent alcohol. The specimens are transferred from a similar grade of alcohol to the chlorine mixture and left until bleached. Depending upon their size and density this period may vary from a few minutes to a day or two.

#### 3. Sulphurous Acid Method.

To a small quantity of sodium sulphate dissolved in water add 2 to 4 drops of hydrochloric acid. Sulphurous acid is evolved and goes into solution.

### IV. Huber's Water-on-the-knife Method for Cutting

A method has been devised by Huber for cutting paraffin sections, using a sliding microtome, with a knife wet with water, just as celloidin sections are cut with a knife wet with alcohol. Using this method it is possible to cut and mount serial sections of embryos or blocks of tissue with greater precision than is possible with the usual dry knife method. Moreover, much thinner sections can be cut with the Huber technique.

<sup>2</sup> Fry, H. J. *Anat. Record*, 34: 245, 1927.



Some details, like those described here, have been added to the method as demonstrated by Huber, but the essential points are as they have been developed by him.

The knife is sharpened by honing first on a yellow Belgian stone, if necessary using water, and finishing on a blue Belgian stone. A hone which has been used with oil should be avoided, since the slightest trace of oil on the knife will prevent the water from extending down to the cutting edge of the knife. For the same reason the knife is not stropped on leather. It is essential that the water does not draw away but is kept in contact with the cutting edge of the knife, otherwise the sections, as cut, will not float upon the water. If the water does not flow down to its edge the knife is cleaned with absolute alcohol and a large camel's hair brush. The alcohol is washed off with water and finally dilute albumen solution is applied. A short segment of rubber tubing slipped over the end of the handle of a brush is a very useful instrument for stroking the edges of the knife, which is thus kept clean and wet. When the knife is locked in position, on the microtome, as much fluid is placed on it as will remain without running off. The lower surface is kept dry. When the knife is drawn across the paraffin block the section will be cut smoothly and accurately but it will roll up unless the corner first touching the knife is held down by the point of a small camel's hair brush. The brush is wet with water and gently stroked over filter paper to bring the hairs to a point, or the brush can be moistened and pointed with the tongue and lips; the latter method is very efficient but has the disadvantage that a few squamous epithelial cells are apt to be carried to the slide. The moist tip of the brush is then slightly pressed against the corner of the block and held there just for a fraction of a second until about the first millimeter of the section is cut, when it is pulled away and the section will then float upon the water. Then with a large camel's hair brush dipped in dilute albumen solution, so that it holds the maximum amount of fluid, the section is lifted up with a rolling motion of the brush and transferred to its proper position on the slip. The latter is best held on a wire frame made to fit in a large shallow Stender dish. The slip should be held at an angle of about  $30^{\circ}$  and a dilute solution of albumen added, to submerge the slide up to the hand for the first row of sections. When these sections have been arranged, enough fluid is removed from the dish to expose a band for the second row and so on until the slip is filled with sections. The level of the fluid in the dish can be easily controlled by placing a second chamber on a stand, provided with a rack and pinion and connecting the two dishes with a flexible syphon. The albumen solution is prepared by adding about 9 drops

of Mayer's egg albumen to 30 c.c. distilled water. The slip is wet with the solution and rubbed with the tubing mounted on the brush handle until the fluid clings to all parts and it is then put in position on the wire frame. As the sections are smooth when cut they require no spreading as ordinary paraffin sections do. The slide is removed from the frame, and the fluid drained off and placed on a warm plate to dry. (C. H. Heuser.)

## D. GENERAL

### I. Alligation

1. **Preparation of Elements in Mixtures.** A convenient method of approximate accuracy for making mixtures of different substances is the following: Write the strength of the solution desired. Beneath this draw a line and at the two ends write the strengths of the solutions to be used. Subtract the smaller figure from the larger in each case and place the subtrahends at opposite ends of the line in reverse position, according to the following diagram:

70	
95	0
70	25

The results thus obtained will give the proportions of the 2 substances. Thus, for example, if it is desired to make the grade of 70 per cent alcohol from 95 per cent alcohol and water (represented by zero), subtracting zero from 70 gives 70. This is placed beneath the 95, the strength of the alcohol. Likewise 70 is subtracted from 95, giving 25, which is placed beneath zero. It is thus indicated that 25 parts of water and 70 parts of alcohol are required to make a 70 per cent solution.

## II. Thickness of Sections

The thickness of sections employed depends largely upon the purpose in view. If it is intended to study the relations of elements to each other in a tissue such as the course of the blood vessels or nerve fibers, sections of considerable thickness are desirable, often up to 50 or 100 $\mu$ . For most histological and cytological purposes sections cut from 7 to 10 $\mu$  are satisfactory. For the finest cytological details sections as thin as 1 or 2 $\mu$  are often helpful. For the best staining results in general, sections should be so thin as to insure cutting through the surface of cells or even of

their nuclei. Otherwise it is difficult to secure accurate detailed differentiation.

### III. Handling Cover Glass Preparations

Many preparations are best made by attaching the material to cover glasses, either in the form of smears or as sections. These delicate pieces of glass are more difficult to handle than thicker slips, but there are devices which make it quite feasible to prepare material in this manner. If it is desired to study both sides of the preparation this may be accomplished by placing it between 2 covers. The method of mounting on cover glasses deserves a wider use than it now has. If only a few covers are to be handled at one time they may conveniently be passed through the different reagents by the use of special forceps designed for this purpose. These are so formed that they rest upon the table conveniently while the cover glasses are immersed in the reagents. In the absence of such special devices the covers may be laid upon small corks and handled in this position, the solutions being placed upon the upturned specimens and removed therefrom by pipettes. In this case care should be exercised to avoid bringing the objects in contact with the edge of the cover which would permit the solutions to be drawn off. Where large numbers of covers are handled at once there are special racks for holding the covers which may be immersed in the various fluids used. One of these designed by Metcalf is constructed of glass and is a very convenient device.

### IV. Preservation of Specimens

If material is to be kept for a considerable length of time there are two general alternatives. It may be run up into 70 per cent alcohol where it, in general, will remain indefinitely without change. In this case there is a necessity of having some means of handling the fluid and this is sometimes a disadvantage. It may be avoided by continuing the operation of infiltration with paraffin if this is the method to be employed for sectioning and in this medium also the material will remain indefinitely without change. The advantages of this arrangement are that the specimens are easily preserved and are in a condition to be immediately sectioned. If it is intended to preserve the material in 70 per cent alcohol, the best method is to place the specimens in small shell vials, stoppered with cotton, and filled with 70 per cent alcohol. These vials are then immersed in some convenient vessel and the whole covered with 70 per cent alcohol. A very convenient container is afforded by some

forms of fruit jars which have lids that are easily sealed tight upon cushions of rubber. The use of corks should be avoided since the alcohol extracts tannin and in some cases injuriously affects the specimens. It is sometimes more convenient to use large shell vials in which a number of specimens are placed with layers of absorbent cotton between them. In this case each specimen of course should have its designating number attached in some manner which will avoid its displacement. If a large number of small vials are kept in one container a very convenient way of placing them for ready access is to stand them on end in rows, or in a spiral, proceeding from the center outward. Thin strips of paper between the rows keep them apart. The numbers of the first and last specimens of the series should be placed upon the outside of the jar. (See Botanical Methods, Chap. iv.)

### V. Tinting Light Colored Specimens for Identification

Objects which have been fixed in Carnoy (p. 558), or sublimate mixtures, are often almost exactly the color of paraffin and are difficult to orient and handle in this case. A very convenient method for avoiding this difficulty is to stain the material lightly and superficially with some bright colored dye like eosin. In case this is subsequently undesirable it may be removed from the tissue by treatment with acid alcohol.

### VI. Air Bubbles

1. **Air Bubbles during Section Spreading.** If sections are allowed to remain too long beneath the lamp the air dissolved in the water will accumulate as small bubbles and will force up the sections above them abruptly and thus disturb the level of section. These bubbles may be avoided by spreading the ribbon more rapidly.

2. **Air Bubbles in Damar.** If the cover glass is properly placed over the sections the damar will be spread uniformly but if it is dropped or lowered unevenly air bubbles will be included. By pressing lightly on the cover glass these may be removed without injury to the section if there is plenty of damar present. However, if the amount of medium is slight and not sufficient to fill the space beneath the cover, large air spaces will be produced. These, of course, cannot be removed by pressure, but sometimes may be replaced by adding damar on the opposite side of the cover, forcing the air out. In the event that these spaces are very large it is safer to remove the cover by placing the slide in xylol and making a fresh effort.

3. **Air bubbles in tissues** arise in various ways. Exposure to the air in passing the material from one solution to another is often responsible for this difficulty, which can easily be avoided by gradual transfer of the specimen from one fluid to the other by substitution. Air bubbles also arise in the process of decalcification where they are, of course, unavoidable. Removal may sometimes be accomplished by placing the material in the fluid underneath negative air pressure. If they are situated deep in the tissue, however, this method may not work. If too much injury is not done the specimen, an opening made by a needle or fine scalpel may make it possible to remove the air.

## VII. Adipose Tissue

A good demonstration of this tissue is afforded by the following process:

Stretch a piece of mesentery containing fat upon rubber rings (see Stretching method, pt. 1). Fix in 5 per cent formalin, rinse in water, stain in a saturated solution of Sudan III and 70 per cent alcohol, five or ten minutes. Differentiate in 70 per cent alcohol until only the fat cells are colored. Wash in water. Counter-stain in dilute Delafield for five minutes. Wash in water. Mount in Farrant's medium (p. 617).

## VIII. Artifacts

As has been intimated elsewhere, it is a question in the minds of some whether all of the figures produced in protoplasm by the use of fixatives are not of the nature of artifacts. In the opinion of those with most experience, however, it seems perfectly evident that the consistency and definiteness of the figures in the best preparations indicate the existence of structures very much of the same character as are present in living material. On the other hand, there are certainly some results following on the application of reagents to protoplasm which are definitely of an artificial character. Some of these have been mistakenly described in the literature as natural structures. Certain crystalline forms resulting from the use of corrosive sublimate closely resemble natural fibrous conditions in the tissues. Also the use of too much Mayer's albumen in mounting sections results in certain coagulation products within open spaces in the tissue which might be mistaken for natural structures. It is important to check up on any unfamiliar or unusual conditions in the sections to see if they may possibly be produced by the technique.

## IX. Feulgen's Stain

It is a serious hindrance to an analysis of cellular constituents that we have no specific chemical tests for them. Thus, while the substance we call chromatin has affinities for certain dyes, none of them can be used as a definite test for it. Recently Feulgen<sup>3</sup> has presented a method which he considers capable of indicating chromatin by a specific reaction. It is carried out as follows:

1. *Fixation.*

Corrosive sublimate 6 per cent aq. sol. ....	98 parts
Glacial acetic acid .....	2 parts

Apparently the P.F.A. mixtures will also serve.

2. *Hydrolysis.*

Sections are exposed for two minutes to the action of cold dilute hydrochloric acid, followed by digestion for four to fifteen minutes in the same strength of acid at a temperature of 60°C.

Hydrochloric acid s.g. 1.19 .....	82.5 c.c.
Distilled water .....	1000.0 c.c.

## 3. Rinse in cold dilute hydrochloric acid.

## 4. Rinse in distilled water.

## 5. Stain in fuchsin-sulphurous acid thirty minutes to two hours.

Although this is a chemical test the length of exposure to the fuchsin-sulphurous acid influences the intensity of the coloration. It is well therefore to vary the length of staining with unfamiliar material.

Basic fuchsin .....	1 gm.
Distilled water .....	200 c.c.

Boil, cool to 50°C., filter, add 20 c.c. of the dilute hydrochloric acid. When further cooled to 25°C., add 1 gm. of anhydrous sodium sulphite. When the solution is decolorized it is ready for use and should be kept in the dark.

## 6. Pass through 3 baths of dilute sulphurous acid.

Distilled water .....	200 c.c.
10 per cent aq. sol. of sodium sulphite .....	10 c.c.
Dilute hydrochloric acid .....	10 c.c.

## 7. Rinse in distilled water.

## 8. Counterstain in light green.

## 9. Mount in damar.

The test indicates nucleic acid (Thymus-nucleic acid) by the presence of an aldehyde group, liberated in its hydrolysis with the dilute hydro-

<sup>3</sup> Feulgen, R., and Rosenbeck. *Ztschr. f. Physiol. Chem.*, vol. 135: 1924.

Feulgen-Brauns, F. *Arch. f. d. ges. Physiol.*, 203: 415, 1924.

chloric acid. Orthopteran spermatocytes treated in this way present essentially the same picture as that following the use of hematoxylin.

#### A RAPID METHOD FOR STAINING EXPOSED SURFACES OR SECTIONS OF THE SPINAL CORD AND BRAIN STEM<sup>4</sup>

This method is particularly adapted for quickly exposing and studying the relations of microscopic structure to surface forms and can be used readily by classes in neuroanatomy. The advantage of the method is the possibility of selecting and cutting through with a safety razor blade and staining in a few minutes to reveal the microscopic details of the parts cut—special regions or levels of the cord and brain stem. Thus one secures the readiest comparison and understanding of the buried microscopic structures and connections which form the basis of surface relief.

Though material from the dissecting room may be used, this naturally varies, the sharpest pictures being obtained from specimens with formalin in the fixative.

The method is not simply one to secure thin sections to demonstrate details, though satisfactory stains of thin frozen sections are secured by this technique. Emphasis is to be laid rather on the advantages of laying open at will and demonstrating quickly, the finer internal relations of the nuclei and connections of any cranial nerve or other special structure of the medulla prominent in surface views, as a helpful preliminary to a later more detailed study of thinner sections, stained either by this or another method.

This technique is an adaptation of the "Rapid Iron Hematoxylin Technique" of Dr. E. C. Cole of Williams College.<sup>5</sup> The following steps are recommended for blocks of tissue or thick sections, the upper cut surfaces of which are specially selected to be stained and studied under a strong light directed from above, rather than by transmitted light from below through cleared sections.

(1) *Material*: Pieces of cord and brain stem, preferably human material from subjects being examined by students, from cadavera in which formalin has been introduced; or fresh animal material which has been hardened for some time. The method also demonstrates areas of degenerated tracts in pathological specimens.

(2) *Cutting*: Sections should be cut free-hand with a slicing stroke by means of a sharp, flat, thin razor blade, the object being to secure a

<sup>4</sup> Section on Staining Exposed Surfaces or Section of Spinal Cord and Brain Stem by Dr. H. McE. Knowler. *Science*, vol. 72: 1930.

<sup>5</sup> Cole, E. C. *Science* (Nov.), 1926.

smooth surface on a block of tissue from 1 to 3 mm. thick, through a particular selected level. Thinner frozen sections stained by this method instead of more elaborate techniques, cleared, and mounted in balsam, may also be used to advantage for special details.

(3) *Mordanting*: Sections should stand in 95 per cent alcohol for five minutes, then transferred to Cole's mordant (made as follows: 50 per cent alcohol, 20 c.c.; ferric chloride, 1 gm.; glacial acetic acid, 2 c.c.). Sections remain in this mordant with upper surfaces covered for at least five minutes. For some material, the mordanting seems sufficient when prolonged until the details of structure have developed and such sections can be removed to alcohol and studied without further treatment, but most material gives better results when stained as follows:

(4) *Staining* (see Cole's article just quoted for full discussion):

First prepare Cole's "stock hematoxylin solution": absolute alcohol, 20 c.c.; sodium hydrosulfite (same as sod. bisulfite) 0.2 gm.; distilled water, 5 drops; hematoxylin crystals, 1 gm. (use light brown, not dark, crystals of hematoxylin).

Now add 5 drops of this stock solution to 10 drops of tap water and follow with 1 drop of ammonium hydroxide.

Before using, allow this mixture to ripen for thirty seconds. Add 5 c.c. of 95 per cent alcohol to this ripened staining solution, and flood the surface of the section which has been taken from its alcoholic bath. Allow the stain to act on the smooth cut upper surface for at least five minutes. This overstains and must be differentiated.

(5) *Differentiation*: This should be accomplished by destaining through the action of 0.4 per cent HCl, the microscopic details of the gray matter being thus brought out sharply in contrast to the darker background. When sharp definition is obtained, rinse off in slightly alkaline 95 per cent alcohol and study by strong reflected light.

(6) *Lighting*: Direct light of strong intensity from above should be used, preferably concentrated on the surface of the section by a condensing lens. Thus lighted, sections can be studied with a hand lens or low-power (48 mm.) objective on a compound microscope.

Of course the mordant and stock solutions will be prepared in advance for class work, and each student will follow the technique in watch glasses and begin study of the specimens in a few minutes. Once stained, sections may be kept in alcohol for later examination.



# THE FUSED QUARTZ ROD METHOD OF ILLUMINATING LIVING STRUCTURES FOR MICROSCOPIC STUDY

MELVIN H. KNISELY

Characteristics of fused quartz rods 632. Historical background 633. Light conduction 636. Light source 638. Temperature control 639.

The following is a rather general description of method and apparatus. The essential parts are the fused quartz rod, which conducts light, and an accompanying glass tube, which conducts fluid to maintain normal tissue temperature. Special light sources and apparatus for controlling the position of a rod, holding it steady, controlling illumination intensity, supporting the specimen and carrying away waste solutions can be selected or designed to integrate with the other features of the experimental procedure involved.

## I. Characteristics of Fused Quartz Rods

Visible light passes by internal reflection through considerable lengths of smooth, transparent, fused quartz rods with but little loss of intensity and no change in color. It follows the contours of the rod around long curves and quite sharp bends, and will be somewhat concentrated where the rod is narrowed.

Fused quartz is transparent also to some bands of the infra red and ultraviolet<sup>1</sup>; however, these wave lengths cannot be conducted through rods as far as can the components of white light.<sup>2</sup>

The thermal conductivity factor of fused quartz is low: about 0.003 20° gram-calories per square centimeter per second per degree Centigrade per centimeter. At room and body temperatures fused quartz reacts very slowly, if at all, with the chemical constituents of tissue fluids.

On account of these properties fused quartz rods are particularly suitable for conducting light to living tissues. The simplest set-up consists in having one end of a rod near a light source, the other end near or touching the tissue to be observed. (Fig. 1.) White or colored light

<sup>1</sup> Wolf, 1928, used a fused quartz rod to irradiate small localized areas of developing eggs and unicellular organisms with ultraviolet light. Its point was drawn out to a few microns in diameter and maneuvered with a standard micromanipulator, and its sides were silvered and lacquered to prevent irradiation of adjacent areas. (Wolf, E. A. An ultra-violet micro-radiator. *Collecting Net*. 3: 20, 1928.)

<sup>2</sup> See Sosman, R. B. The properties of silica. *Am. Chem. Soc. Monograph Ser.*, No. 37. Chemical Catalogue Co., 1927. See Chap. xxxvi for absorption theory and data.

can be conducted from a suitable source, through long rods if necessary and around obstacles which would interfere with the straight-line propagation of light by lens and mirror systems, to the spot to be illuminated. By use of appropriate rods and light sources many tissues and organs of small living animals (such as frogs, mice, kittens, etc.) can be brilliantly illuminated for microscopic study. Proper design and use of the rods reduces the surgical procedure necessary for observing internal organs and makes it possible to study them in, or nearly in, their normal locations with their vessels, nerves and attachments intact. As a rod occupies little space it is easy to illuminate a specimen which is surrounded by intricate apparatus.

By controlling the position of a rod's light delivery tip the light can be delivered either by reflection, or at a "low incident angle" (compare Vonwiller<sup>3</sup>), or by transillumination. The light is sufficient to transilluminate relatively opaque or thick translucent structures.

For aseptic techniques the rods can be steam sterilized, flamed, or washed in the usual bactericidal solutions.

## II. Historical Background

Fused quartz is not the first material used to conduct light to living structures. Basler<sup>4</sup> designed a method of illuminating internal organs with a curved rod of glass. Loeffler and Nordman<sup>5</sup> used Basler's method in their study of the mammalian liver circulation. Basler's description includes a mathematical presentation of the phenomenon of internal reflection which applies to fused quartz if its indices of refraction and critical angles are substituted in the equations. (Sosman gives the indices of refraction of fused quartz for many wave-lengths of the visible spectrum.) However, fused quartz is superior, as glass is less transparent and permits only short lengths to be used. Glass absorbs some wave-lengths more than others, so that the color of the light delivered to the tissue is not the same as that sent into a glass rod, and light does not follow around sharp bends in glass in usable amounts.

Florey and Carleton<sup>6</sup> spread the mesentery out over a glass plate and

<sup>3</sup> Vonwiller, P. *Histologische Methoden und Ergebnisse der Mikroskopie im auffallenden Licht*. In: *Handbuch der biologischen Arbeitsmethoden* Herausgegeben von Geh. Med.-Rat. Prof. Emil Abderhalden. Berlin, Urban and Schwarzenberg, 1920.

<sup>4</sup> Basler, A. Über eine neue Methode zur mikroskopischen Untersuchung innerer Organe des Lebenden Tieres im durchfallenden Licht nebst dem Versuch einer Theorie der das Licht leitenden Glasstabe. *Pfleuger's Arch.*, 167: 228-244, 1917.

<sup>5</sup> Loeffler, L., and Nordman, M. *Leberstudien*. *Virchows Arch.*, 257: 119-181, 1925.

<sup>6</sup> Florey, H. W., and Carleton, H. M. Rouget cells and their function. *Proc. Roy. Soc. London*, s. B, 100: 23-31, 1926.

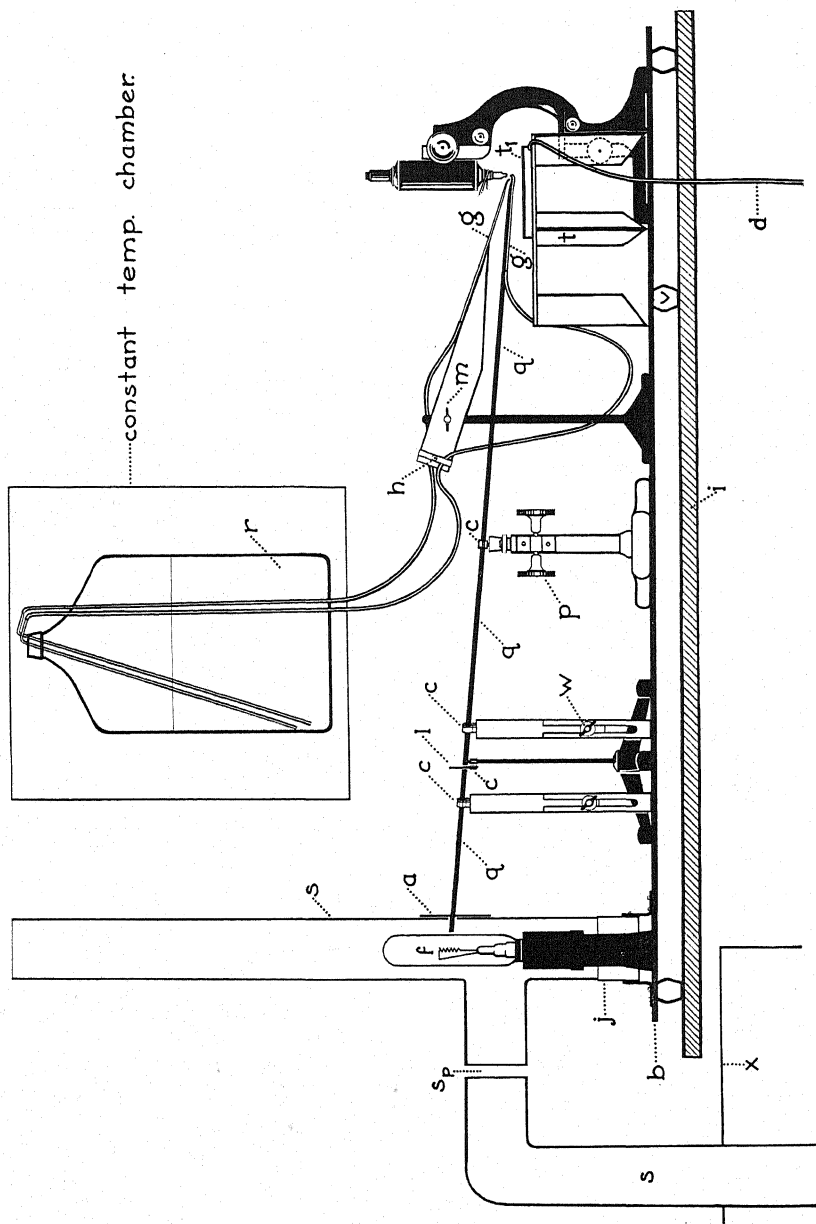


FIG. 1

illuminated the area to be studied by means of an internal reflecting rod. They do not name the material of which the rod was made. Leiter<sup>7</sup> used a curved fused quartz rod to illuminate microscopic objects, because the passage of light through the rod eliminates the image of the lamp filament. Silverman<sup>8</sup> described the use of curved fused quartz rods to furnish "cold light" for microscope use. (The term "cold light" is misleading, however, since a fraction of the light energy which strikes an object is transformed into heat.) Wearn et al.,<sup>9</sup> in the study of the circula-

<sup>7</sup> Leiter, S. B. Microscope illumination by means of quartz rod. *J. Optical Soc. Am.*, 11: 187-189, 1925.

<sup>8</sup> Silverman, A. Cold light for the microscope. *J. Ind. Eng. Chem.*, 17: 573, 1925.

<sup>9</sup> Wearn, J. T., Ernstene, A. C., Bromer, A. W., Barr, J. S., German, W. J., and Zschiesche, L. J. The normal behavior of the pulmonary blood vessels with observations on the intermittence of the flow of blood in the arterioles and capillaries. *Am. J. Physiol.*, 109: 236-256, 1934.

FIG. 1. Accessory apparatus for one form of tissue illuminator. *a*. Asbestos sheet, containing hole which fits around rod. *b*. Base, 5/16 x 24 x 30 in.\* *c*. Clamps, made of spring clothes pins. *d*. Drain tube to carry waste fluid from drip tray. *f*. Filament of 400-watt "T 10" bulb. *g* and *g*. Glass tubes which bring Ringer's solution to tissue. *h*. Screw clamp regulating flow of Ringer's solution in one tube. *i*. Top of work table. *j*. Joint in stove pipe. *l*. Light filter held in break in rod by a clamp in movable stand. *m*. Movable stand controlling upper glass tube tip. *g*. *p*. Pinion and rack of old dissecting microscope to control elevation and lateral movement of tip of rod. *q*. Fused quartz rod, with concentrating segment near delivery tip. *r*. Ringer's solution. *s*. Stove pipe. *sp*. Small space to prevent transmission of vibration from fan to apparatus on base. *t*. Three-legged table, acting as microscope stage. *t*. Drip tray. *v*. Rubber stopper, used as leg to isolate base from vibrations. *w*. Wing nuts to adjust position of clamps holding rod. *x*. Box acting as compression chamber.

\* For portable apparatus the base *b* is of masonite fiber board, heavily shellacked to protect it from spilled solutions; to diminish vibrations, for research, it should be of heavy boiler-plate iron.

If the drain tube, *d*, leads to a large (about 5 gal.) bottle which is partly evacuated by a tube from a suction filter aspirator, the waste fluids are removed from the drip tray automatically. Excellent drip trays, *t*, are made from small hard-rubber photographic developing trays, by cutting off the rims with a hack saw, to a height of 1 cm. For attaching the drain tube, fasten a bent glass tube in one corner with de Khotinsky cement, so that the inside end is close to the floor of the tray.

The table, *t*, is made of well-shellacked masonite, and has one leg about 5 mm. shorter than the others to tilt the top toward the outlet of the drip tray. By having the specimen on a table the microscope can be moved about to get different views of various parts. The microscope has its stage removed.

An electric fan blows air into the box, *x*. The air sent into the box is vibrating due to the forward "pats" given it by the blades at a certain position as they pass by. The box is large enough (1/3 c.m. or more) so that air comes out in a steady stream; thus the vibration of the air is prevented from shaking the light bulb.

tion of the living mammalian lung, used a straight truncated cone of fused quartz to conduct light to lung tissue.

Some details of the method described in this article are given more fully elsewhere (Knisely<sup>10</sup>).

### III. Light Conduction

Because there are some types of fused quartz rods which do not conduct light, in ordering rods the smooth, optically clear, machine-made ones should be specified.<sup>11</sup> They may be of nearly any diameter; a serviceable size for almost any internal organ of mice or frogs is a quarter inch (6-7 mm.). These are not broken easily and are inexpensive.

In this description the parts of a rod are termed (1) the base, the end that receives light, (2) the main conducting piece, which may consist of two parts as in Figure 1, a short "lamp segment" near the bulb and a longer segment near the object, (3) the concentrating segment, a narrowed length of the conducting piece, which concentrates the light, and (4) the delivery tip, which directs light to the field of observation.

For intense illumination a single solid rod containing one or more concentrating segments serves best. There are, however, several advantages in having the light-conducting system made in two or more pieces, placed end to end. This permits various interchangeable units consisting of a concentrating segment and delivery tip to be used on one shaft. A snug ferrule (made by slitting a short brass tube from end to end) holds the delivery tip unit firmly to the shaft so that the two can be mounted and manipulated as a single piece.

Frequently it is imperative to vary the illumination of a specimen without disturbing it mechanically. If a system has a joint as at *l* in Figure 1 and the two parts are supported independently, moving the lamp segment more or less out of alignment with the shaft controls the amount of light entering the latter, without touching it. Light filters can be inserted here without disturbing an experiment. A number of structures which cannot be distinguished from their surroundings during the use of white light become conspicuous in colored light. For instance, frog red-cell nuclei and the brush-border lining of frog kidney tubules stand out well in some types of green light. The end of a rod which re-

<sup>10</sup> Knisely, M. H. A method of illuminating living structures for microscopic study. *Anat. Rec.*, 64: 499-523, 1936.

<sup>11</sup> These fused quartz rods can be purchased from Amersil Co., Hillside, N. J., or from Thermal Syndicate, Ltd., Brooklyn, N. Y., or at Wallsend-on-Tyne, their English office.

ceives light or sends it across joints should be a clean break, prepared by nicking with a file and snapping it off (just as one cuts a glass tube). This end should be left without grinding or fire polishing.

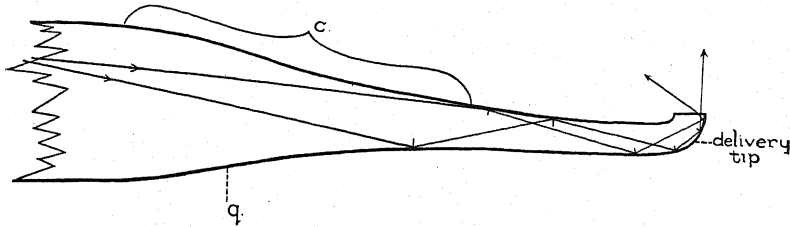


FIG. 2. One form of light delivery tip. Two rays of light are shown. Note that they leave tip at different angles. Light from tip is diffused, not focused. Bracket (c) marks position of a "concentrating segment."

The concentrating segment is prepared by drawing out the end of the quartz rod (Fig. 2). The shape of the "draw" determines the efficiency of this portion to concentrate light. The draw should not be a conical taper, but should start as a sudden, quick taper and then trail into a long, gradually narrowing shank. (For more detailed discussion, see Knisely.<sup>12</sup>) For very intense illumination a lamp segment of large diameter can be used with a concentrating segment directing light into a narrower shaft.

Delivery tips can be made in various shapes. A tip having a shank 3 to 5 cm. long and 1 to 3 mm. in diameter with a single bend at the end (Fig. 2) is serviceable for many purposes. After laparotomy such a tip can be slipped under a loop of intestine, the edge of the liver, or a frog's lung or mesonephros. By inserting the tip in the mouth of a frog placed ventral side down and removing the skin and dorsal bones of the skull one can study the more superficial parts of the transilluminated brain. Such a tip can also be supported above an organ to give reflected light, or the light can be directed across a specimen at various angles. Terminal bars between cells sometimes stand out very clearly in "low incident light."

The capacity of the tissue to scatter light limits the depths to which one can resolve structures in the transilluminated parts of organs. Structures can be revealed farther below the surface of spleen and liver than that of smooth muscle, and farther into relaxed than contracted smooth muscle. Low-power binocular dissecting microscopes can be focused quite deeply. With the compound microscope observations must be nearer the

<sup>12</sup> Knisely, M. H. *Anat. Rec.*, 64: 499, 1936.

surface. Structures the size of liver sinusoids, kidney glomeruli and proximal convoluted tubules of frogs, including their brush-border linings, can be studied with initial magnifications up to  $\times 40$  (using a Zeiss "D" long focus  $\times 40$  water-immersion objective). Higher initial magnifications greatly restrict the working distance so that observations are limited to structures close to the surface. The light coming from a delivery tip is diffuse, not focused like that from a sub-stage condenser, which limits the effective resolution of higher powered objectives.

The light-emitting surface of a delivery tip can be either fire-polished or ground, like a ground glass surface. The smoothed edges of the former minimize injury to delicate structures. For most work, however, the ground glass type (Fig. 2) is preferable since it acts almost like a primary light source. Such a surface can be made by grinding a tip on a motor-driven oil stone or on a glass-blower's steel grinding disc, coated with a small amount of wetted No. 600 carborundum powder. If the grinding disc is used, the quartz tip should be held very lightly against the disc, for it grinds rapidly.

Only a part of the light sent into the base of a rod reaches the specimen. How large a fraction is lost depends upon the shape of the rod and the cleanliness of its surface. Some light is lost at curves, bends, concentrating segments and joints, at surface wrinkles and at soiled spots, particularly finger prints. Hence, no more bends and joints are used than are necessary, wrinkles are kept at a minimum by careful glass-blowing and the rods are kept clean by frequent scouring with soap and scouring powder, rinsing thoroughly in a stream of distilled water and drying with a lint-free cloth. By selecting adequate light sources enough light can be sent into the base of a rod so that, regardless of the losses, more than enough leaves the delivery tip for most purposes.

#### IV. Light Source

Lamps manufactured for home motion-picture projection, "T 10" medium prefocused base,<sup>13</sup> are excellent sources of intense white light. The "T 10" is a trade term which indicates a cylindrical bulb  $1\frac{1}{8}$  inches in diameter. "T 10" bulbs with 200, 400 or 500 watt tungsten filaments can be purchased. According to Coblenz,<sup>14</sup> tungsten filaments in glass bulbs emit very little ultraviolet light beyond  $320\mu\mu$ . (Campbell

<sup>13</sup> "T 10" medium prefocused base bulbs can be purchased from General Electric Co., Chicago, or Westinghouse Electric Supply Co., Chicago. Medium prefocused base sockets can be obtained from Bryant Electric Co., Chicago.

<sup>14</sup> Coblenz, W. W. Sources of radiation and their physical characteristics. *J. A. M. A.*, 95: 411-413, 1930.

and Hill<sup>15</sup> found that ultraviolet light from carbon arcs placed 60 cm. from tissue produces pathological dilatation and stasis in capillaries.)

These lamps while incandescent are cooled by enclosing in 3 in. stove

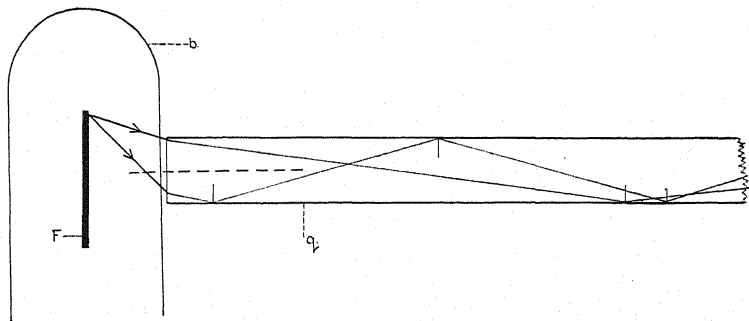


FIG. 3. Path of two rays of light from one end of filament (F) as they pass through glass bulb (b) into fused quartz rod (q).

pipe 24 in. high, through which air passes. A 200 watt lamp can cool itself by convection if the pipe is mounted so that air enters freely at the bottom. More powerful lamps should have air driven past them by an electric fan. (See the legend of Fig. 1 for methods of keeping the vibrations of the fan and of the air from reaching the rest of the apparatus.) These lamps use the standard 115 volt current. However, the life of a lamp can be considerably prolonged by operating it at a little below its rated voltage. For most purposes, 400 watt lamps are preferable.

The base of the quartz rod is placed against the bulb, directly opposite the filament (Fig. 3) which forms a pattern about 1 cm. square. Pre-focused base bulbs and sockets are used in order to maintain the plane of the filament approximately perpendicular to the long axis of the rod. A light ray which falls on the base of the rod is refracted toward its axis. Thus rays within the rod strike its inside surface at an angle greater than the critical angle of fused quartz in air, and so are reflected back into the rod again. The advantage of the slender "T 10" bulb is that the base of the rod is but  $\frac{5}{8}$  of an inch from the filament and consequently collects light of high intensity.

## V. Temperature Control

In order to control the temperature of illuminated living structures the three methods by which heat may be transferred to or from an ob-

<sup>15</sup> Campbell, A., and Hill, L. The effects of light upon leucocytes and blood-vessels in the mesentery of the living animal. *Brit. J. Exp. Path.*, 5: 317-327, 1924.



ject, i.e., conduction, convection and radiation, must be taken into consideration.

Owing to the low thermal conductivity of fused quartz a rod does not conduct heat to or from a tissue rapidly enough to affect its temperature appreciably. Using rods 30 to 75 cm. long or longer places the hot light source far enough away from the specimen so that convected or radiated heat does not reach it.

But it is impossible to illuminate a non-transparent structure without at the same time heating it. The color of an object, even a translucent object, as seen by either transmitted or reflected light is due to the wave-lengths which reach the eye after part of the incident light is "absorbed" by that object, that is, transformed into heat. Filters as commonly used between light source and illuminated object can shelter a specimen from direct heat radiation, and reduce the intensity of some of the incident wave-lengths, but they do not alter the fact that a part of the light which passes them is transformed into heat by the specimen.

In continuously illuminating a living object, heat is concomitantly applied to it at a constant rate. If the object is small, thin and very nearly transparent, and if its illumination is relatively dim, this continuously produced heat may be transferred to adjacent objects so rapidly that the temperature of the object never rises enough to interfere with its normal functioning. However, in illuminating relatively thick, translucent structures brightly enough for study with a microscope, heat is developed faster than it can be removed without assistance. To remove this heat a *flowing* solution at constant temperature is applied to the tissue by two glass tubes (*g* and *g* in Fig. 1). On account of the high "specific heat" of water the flowing solution can absorb the heat as fast as it is produced with but little change in its own temperature. One of the glass tubes is fastened to the fused quartz rod by means of small bands cut from rubber tubing. This tube is adjusted to direct its flow across the surface of the delivery tip (Fig. 4) between it and the tissue.<sup>16</sup> The fluid from the other glass tube is directed over the remainder of the exposed surface of the organ being studied, or across the surfaces of other exposed structures. A centimeter or two of very fine rubber tubing (1 mm. outside, 0.5 mm. inside diameter<sup>17</sup>) may be slipped part way

<sup>16</sup> The use of a recently developed type of fused quartz rod, which delivers the Ringer's solution through its own hollow tip, eliminates the necessity for the accompanying glass tube so that a smaller incision can be used, and insures the delivery of the solution to the area illuminated. Such rods can be obtained from James Graham, University of Pennsylvania Medical School, Philadelphia, Pa. A description of them will be submitted for publication to the *Anatomical Record*, probably in 1937.

<sup>17</sup> This slender tubing is obtained from the Miller Rubber Co., Akron, Ohio.

onto the ends of these tubes, making soft tips to prevent injury of delicate structures.

The fluid delivered to a tissue should be isothermal and isotonic with

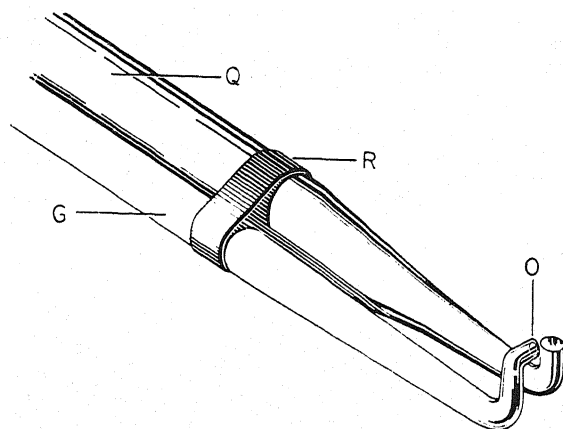


FIG. 4. O, Orifice of glass tube (G), fastened to rod (Q) by small rubber band (R). Tube can be any shape as long as Ringer's solution is directed across end of delivery tip.

the fluid which normally bathes it. Thus, if the tip of the rod is placed in a frog's mouth, the accompanying tube supplies water at room temperature. If the rod is inserted in a frog's body cavity, amphibian Ringer's solution at room temperature is used. For mammals it is imperative that the temperature of the mammalian Ringer's solution where it comes in contact with the tissue be maintained at that normal for the organ being studied. The following factors assist in doing so: (1) The use of a large constant temperature chamber to hold a 5 gallon bottle of the Ringer's solution. The chamber should be supplied with (a) a good thermoregulator,<sup>18</sup> operating a relay<sup>19</sup> which controls the electric heating elements, as the large current in the latter circuit would injure the thermoregulator; (b) a fan (hung from the top of the chamber by straps of sponge rubber to isolate its vibrations) to circulate the air in the chamber; (c) electric heating elements having a high heat-producing ability and a low heat storage capacity,<sup>20</sup> so that the temperature of the

<sup>18</sup> The vapor pressure type thermoregulator No. 500A, from The Chicago Surgical and Electrical Company, Chicago, is suitable for this purpose. It can be adjusted to maintain any temperature from 32° to 42°C., with a variation of not more than  $\pm 0.5^\circ$ .

<sup>19</sup> Small rugged relays are made by Ward Leonard Electric Co., Mount Vernon, N. Y. I use their "single pole, single throw, double break, normally open" 60 cycle, 6 amp., 106-212 volt type.

<sup>20</sup> The Chicago Surgical and Electrical Company makes a new type of woven wire heating element especially for constant temperature chambers.

air in the chamber will react instantly to the turning on or off of the current. Several small heating elements distributed around the chamber, each slightly heating its area, keep the temperature of the chamber more uniform than a single large heating element; and (d) sheet asbestos shields between the heating elements and the stock bottle so that direct heat radiations will not raise the temperature of the solution above that of the surrounding air. The shields should not touch either the heating elements or the bottle, and air should circulate freely about them. (2) The stock of warm solution should be large so that its temperature will change but imperceptibly during the slight (not more than  $\pm 0.5^{\circ}\text{C}.$ ) rapid (at least once each two or three seconds) fluctuations in the temperature of the air in the chamber. (3) The warmed solution should be put in the constant temperature chamber early enough so that its temperature may come into equilibrium with that of the air in the chamber. (4) Because the temperature of the solution drops as it passes down the tubes leading from the bottle, that of the chamber must be one or two degrees higher than that of the organ studied. To reduce this drop the tubes should be short and well insulated.

It is essential that the rate at which the wash solutions leave the glass tubes be carefully controlled, for if the flow is too slow the heat developed at the tip of the illuminator is not carried away as fast as it is produced. If the flow is too rapid the beating of the wash solution against the tissue frequently initiates a hyperemia.

When working with a water-immersion lens it is usually advantageous to place the upper glass tube tip against the side of the field lens of the objective and let the fluid flow gently down across it over the tissue. The force of the stream is thus spent against the objective rather than against the specimen. The film of solution which is maintained between lens and tissue acts as the immersion fluid.

The bodies of mammals are kept warm by blanketing with dry non-absorbent cotton. Wicks of absorbent cotton conduct the used wash solution into the drip tray, so preventing it from wetting the cotton blanketing.

Amphibian Ringer's solution in contact with an amphibian's skin affects its water balance which in turn affects some circulatory processes. To prevent this a gentle flood of plain water may be directed against the outside of the animal in order to dilute the Ringer's solution as it drains from the site of an incision.

# THE MICROINCINERATION METHOD OF DEMONSTRATING MINERAL ELEMENTS IN TISSUES

GORDON H. SCOTT

General considerations 643. Microincineration method for demonstrating localization of total minerals 646. Quantitative measurement of mineral residue of tissue sections 661.

## I. General Considerations

It is quite natural to expect that any experimental procedure applied to living tissue will alter conditions existing in the normal animal. Vital dyes, innocuous as they seem to be, are not a constituent of the normal cell and their very presence renders their host a thing odd among its fellows. Of these delicate changes brought about within its cells the whole organism may not be aware. No expression of unbalance may reach the surface in such magnitude that man can observe and record it with the instruments at his disposal. That such is the state of affairs makes the task of the histochemist a difficult one. He must make the attempt to explain cellular phenomena on the basis of methods which are wholly unsuited to the maintenance of cellular integrity. He should keep constantly in mind the fact that histochemical techniques are of themselves difficult. They must reveal beyond doubt the substance in question. Furthermore, the element sought must remain in the same topographic relationship that obtained in the living tissue; otherwise nothing more is accomplished than is possible with ordinary biochemical analyses. Even if the substance is altered in its intracellular topography, little has been gained. The extremely minute quantities of elements present in a section of tissue prepared for study serve further to harass the experimenter. Ordinary microchemistry is by comparison the dealing with gross quantities of material. It is safe to say that the calcium, for example, present in a section of tissue thin enough to permit cytological study transferred into the test tube, freed from all the organic and inorganic "contaminants" normal to body tissues, would still tax the ingenuity of a skilled chemist. Then, too, one must rely on color differentials or crystal shape for diagnosis. Frequently, a tinctorial reaction may be sufficiently intense for macroscopic observation yet utterly useless for a histochemical test, because of lack of character when reduced in quantity and viewed with a bright source of light, conditions necessarily imposed by the nature of the study.

The techniques commonly employed for the demonstration of inorganic salts and metals in cells and tissues are of all histochemical tests the most capricious since they frequently involve the use of strong acids which dissolve the minerals and of bases which may deposit or precipitate them in quite different localities than those in which they existed in the living cell. Many of the solutions used to treat fresh tissue or frozen sections are strongly hypertonic and would therefore have every tendency to encourage the diffusion of ions through nuclear and cell membranes. Subsequently such ions might come to rest in any region other than the one originally occupied by them. Another no less important consideration involved in histochemical procedures, and pointed out by Policard,<sup>1</sup> is the adsorption of reagents by the complex colloidal surfaces present in protoplasm. For example, many histochemical reactions depend upon a succession of treatments with reagents which produce the final characteristic color or crystal shape. If previously used chemicals are not thoroughly removed from the cells by washing, the reaction may take place diffusely throughout the tissue. In such a colloidal system as protoplasm, electrolytes cling tenaciously despite many washings and must of necessity be considered as being present and vitiating to some extent the subsequent reaction. Any method which is free from these objections is of considerable value in the localization of tissue salts.

Much has been written about the delicacy, the specificity and general neatness and dispatch with which a histochemical reaction reveals a sought-for salt. Little time has been devoted, however, to a discussion of the changes in salt localization by diffusion which must inevitably occur when tissues are subject to the ordinary fixation process. Even the coagulation of tissues does not insure against this happening. Perhaps the coagulated proteins with their inorganic radicals are truly fixed but it is unlikely that the free salts are rendered immobile.

It would seem then that with our present technical methods of handling tissues we are indeed far from arriving at truths in cell chemistry. We have techniques which are free from some of the objectionable features, yet in the main these do not give us the information which we so keenly desire. It seems best to say that with a goodly number of our procedures the best that can be adduced from the pictures they yield is that in sets of experimental tissues a definite configuration is produced, but one which can, by no stretch of the imagination, be considered as that obtaining in the living. It is most unfortunate that such is

<sup>1</sup> Policard, A. Harvey Lectures, 1931-1932. Balt., Williams and Wilkins, Ser. 27, 1933, p. 204.

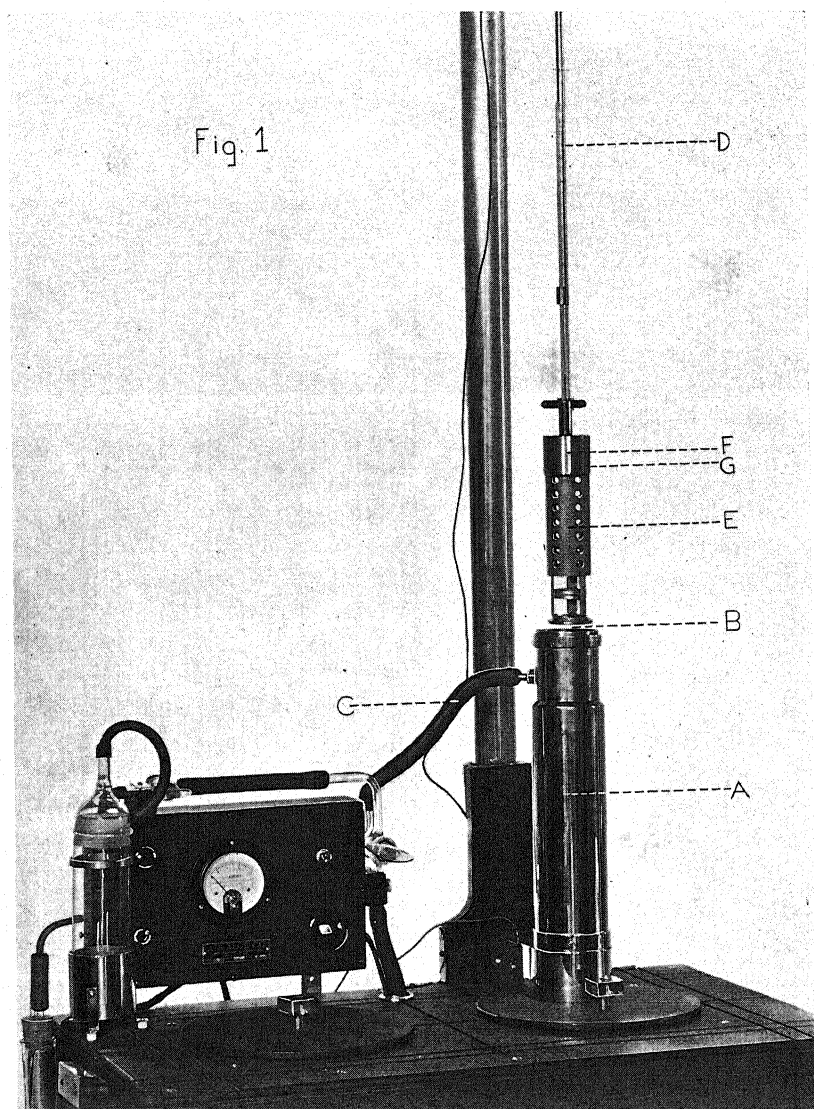


FIG. 1. Scott and Williams' cryostat showing vacuum chamber open and tissue container exposed. A, Metal vacuum chamber. B, Tapered joint vacuum seal. C, Connection to vacuum pump. D, Steel supporting tube connecting alcohol chamber with mercury regulator. E, Copper-walled tissue chamber. F, Alcohol expansion chamber connecting outside vacuum system with mercury regulator through D. G, Heating coil around alcohol chamber.

the case but we must be content until methods of tissue handling are devised which will meet both theoretical and practical objections.

The following exposition of methods is confined to those which in the author's opinion are the best extant. They are presented with all the points just brought out in mind, and with the realization that with few exceptions they fall short of the goal histochemistry should set for itself.

## II. Microincineration Method for Demonstrating Localization of Total Minerals in Cells and Tissues

1. **Fixation of Tissue for Microincineration.** It is essential that a fixative be selected which neither adds minerals to the tissues nor removes them. Fixatives containing chromium or mercury salts are to be avoided as are those which contain strong acids. Absolute alcohol is naturally the fixative of choice because very few of the inorganic constituents common to protoplasm are soluble to any extent in this fluid. But absolute alcohol is a notoriously poor fixative since it causes extreme shrinkage of tissues and loss of structural detail. Theoretically, formaldehyde possesses certain properties which are inimical to the method; but, practically, little difference can be seen in the mineral content of tissues fixed in an alcohol-formalin mixture and those fixed in absolute alcohol alone. Neither copper sulphate nor calcium chloride can be used to dry the alcohol used for fixation. Though these substances settle in the bottom of the container, the supernatant fluid has in it a large number of extremely minute particles of the chemicals, as evaporating a drop of it on a clean slide and subsequently examining it with dark-field illumination will readily prove.

The possibility of salts passing from the tissues into the fixative must be considered. Policard and Okkels<sup>2</sup> found that emulsions of dried meat powder lost 10 to 14 per cent of their total mineral content when treated with solutions of alcohol, toluene, or amyl alcohol. The conditions governing these experiments make their interpretation in terms of actual practice difficult. The emulsions were made from desiccated meat powder and water spread upon glass slides, dried, and then extracted with the solvents selected. Naturally, salts of the dried and broken-up muscle cells are left in their most readily soluble form instead of being in an environment of precipitated proteins reinforced by coagulated cell membranes which renders them difficult of solution.

The experiments of Scheid<sup>3</sup> on microincineration of brain tissue indi-

<sup>2</sup> Policard, A., and Okkels, H. *Anat. Rec.*, 44: 349, 1930.

<sup>3</sup> Scheid, K. F. *Virchows Arch.*, 277: 673, 1930.

cate that extraction of phospholipoids before ashing is a necessary step in the procedure. He points out that the possibility of loss of inorganic salts by treatment with the extractives always exists but that in all probability it is not as great as Henkel<sup>4</sup> supposes. Considerable experience with incineration of nervous tissue (brain, spinal cord and the attached ganglia) has demonstrated that the extraction process is not required. Excellent cytologic pictures have been obtained with alcohol-formalin fixation. It is, however, essential to have thin sections, 3-5 microns, to secure good results. As indicated by Scheid<sup>3</sup> sections which are 10-15 microns in thickness incinerate slowly and imperfectly if the phospholipoids are not previously extracted.

The Altmann method of freezing with liquid air and of subsequent drying in vacuo, recently developed at the University of Chicago by Gersh,<sup>5</sup> Bensley,<sup>6</sup> and Hoerr,<sup>7</sup> has proved to be valuable in the preparation of tissues for microincineration. In contrast to other fixatives, coagulating agents are avoided, yet the sections secured show many cytologic details and are suitable for various microchemical reactions. It seems, however, that there is some denaturation of proteins following freezing and thawing of tissue juices (Finn<sup>8</sup>) and probably also under the conditions imposed by Gersh's method. Furthermore, this author's statement that the salts of the tissues, which are of primary interest in microincineration, are not altered can be questioned on the grounds of experience with behavior of solutions of salts at low temperatures. The temperature of liquid air is of course sufficiently low to maintain equilibrium in the frozen system, but the apparently arbitrarily chosen temperature of  $-20^{\circ}\text{C}.$ , which is kept constant during dehydration, is above the eutectic point ( $-21.2^{\circ}\text{C}.$ ) of even such a universally distributed substance as sodium chloride. What probably happens, then, during the process of fixation as detailed by Gersh, is that the freezing by liquid air produces a solid mass of ice crystals and crystals of salts, inorganic and organic (for example, protein salts of metals), in equilibrium; as this mass is warmed above the eutectic point the established equilibrium is disturbed and there occurs a partial dissolution of ice crystals and crystals of salts with a practical likelihood of subsequent diffusion and shift in the location of the ions as dehydration takes place. A lower temperature (below  $-54.9^{\circ}\text{C}.$ , the eutectic point of  $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$ ) would satisfy this objection but makes the method unwieldy as the time required for de-

<sup>4</sup> Henkel, K. O. *Handb. d. biol. Arbeits-Methoden* (Abderhalden), 5: 1471, 1929.

<sup>5</sup> Gersh, I. *Anat. Rec.*, 53: 309, 1932.

<sup>6</sup> Bensley, R. R. *Anat. Rec.*, 58: 1, 1933.

<sup>7</sup> Hoerr, N. L. *Anat. Rec.*, 65: 293, 1936.

<sup>8</sup> Finn, D. B. *Proc. Roy. Soc.*, s. B, 111: 396, 1932.



Fig. 2

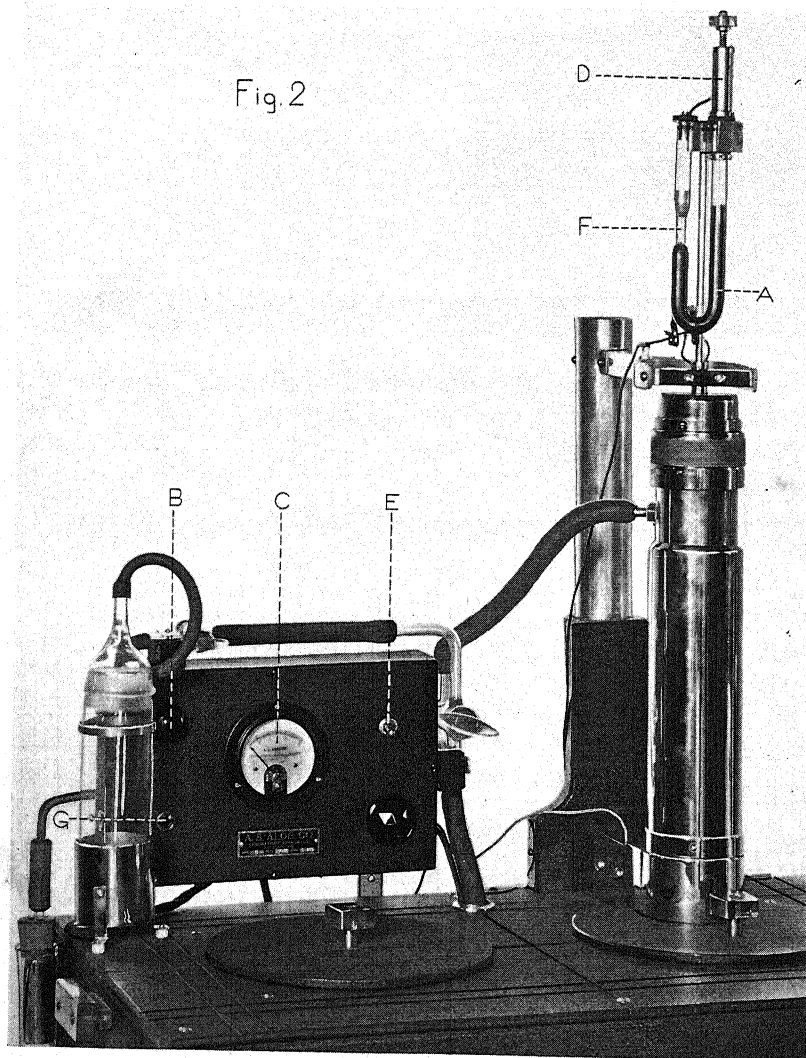


FIG. 2. Cryostat with vacuum chamber closed and arranged as for operation. A, Mercury regulator. B, Rheostat for regulating heat input to coil. C, Ammeter for heat regulation control. D, Syphon tube for adjusting thermostat. E, Switch for heating coil. F, Capillary tube of mercury regulator.

hydration is enormously lengthened due to the very low vapor pressure of ice at this temperature.

Experiments in this laboratory show the distribution of inorganic salts in incinerated tissues, prepared as described by Gersh except that the temperature of dehydration was at  $-28^{\circ}\text{C}$ .,  $-33^{\circ}\text{C}$ . and  $-78.5^{\circ}\text{C}$ ., respectively, to be markedly different from similar tissues dehydrated at  $-20^{\circ}\text{C}$ ., as originally advocated. These preliminary experiments seem to indicate that the objection to dehydration at  $-20^{\circ}\text{C}$ . is more than a theoretical one and that despite the technical difficulties involved, it is a step in the right direction. Lower temperatures as suggested by Scott<sup>9</sup> have been adopted by most of the workers employing the frozen dehydration method (Goodspeed and Uber,<sup>10</sup> Bensley and Hoerr,<sup>11</sup> Hoerr,<sup>12</sup> Gersh<sup>13</sup>). But it is well to remember, in this connection, the investigations of Chambers and Hale<sup>14</sup> on the formation of ice in protoplasm. According to these authors, there is a different rate of freezing in living tissues and the cell membrane acts as a barrier to ice formation. It would seem probable that even sudden cooling with liquid air might not establish an instantaneous equilibrium throughout the tissue. Their experiments indicate a lag of  $10^{\circ}\text{C}$ . between the freezing of the exterior and the interior of muscle fibers. Plant cells exhibit a similar phenomenon. It is to be noted that Chambers and Hale mention as one of the factors which tends to prevent the freezing of the interior of the living cell, dehydration which increases the total and balanced salt concentration of the interior. It is doubtful if the immersion of tissue in liquid air, despite its low temperature, results in as sudden freezing as might be supposed since the protective layer of gas instantly formed around the warmer substance tends to inhibit the escape of heat. Slices of organs plunged into absolute alcohol, cooled to its freezing point, harden more rapidly than do similar tissues in liquid air. This method of freezing of tissues has been found to achieve good results. The penetration of alcohol into the tissue is prevented by a protective layer of ice and the cooling substance evaporates very soon after the reduction of pressure in the vacuum system. Better yet is the use of iso-pentane (practical) as recommended by Hoerr<sup>15</sup> since it does not freeze at liquid air temperature, thus giving a more rapid freezing rate than alcohol.

<sup>9</sup> Scott, G. H. *Protoplasma*, 20: 133, 1933.

<sup>10</sup> Goodspeed, T. H., and Uber, F., *Proc. Nat. Acad. Sc.*, 20: 495, 1934.

<sup>11</sup> Bensley, R. R., and Hoerr, N. L. *Anat. Rec.*, 60: 251, 1934.

<sup>12</sup> Hoerr, N. L. *Anat. Rec.*, 1936. In press.

<sup>13</sup> Gersh, I. J. *Pharm. & Exp. Ther.*, 52: 231, 1934.

<sup>14</sup> Chambers, R., and Hale, H. P., *Proc. Roy. Soc.*, s. B, 110: 336, 1932.

<sup>15</sup> Hoerr, N. L. *Anat. Rec.*

Since for many reasons the apparatus described by Gersh<sup>16</sup> was impractical for use in problems dealing with mineral salts a cryostat more simple in operation, yet which permitted a greater latitude of temperature control, was devised by Scott and Williams.<sup>17</sup> It seemed highly desirable to have an instrument which would be easy to operate, made of metal and susceptible to wide control of temperature range. The apparatus is shown in Figures 1 and 2.

It consists of a vacuum-tight metal cylinder (Fig. 1, A) of suitable dimensions which is fitted with a tapered joint top (Fig. 1, B) and is connected by rubber pressure tubing (Fig. 1, C) to a Cenco Hyvac pump. The top supports, by a long steel tube (Fig. 1, D), a tissue chamber (Fig. 1, E) of heavy walled copper pipe. The upper quarter of the tissue chamber is sealed off from the bottom and forms a container (Fig. 1, F) which is filled with alcohol. This alcohol chamber is continuous with the supporting tube and connects with a mercury regulator (Fig. 2, A) outside the vacuum system. Wound about the alcohol chamber, in grooves cut in the copper, is a length of fine resistance wire (a heating coil) (Fig. 1, C) which is connected in a simple circuit with a rheostat (Fig. 2, B), milliammeter (Fig. 2, C) and the mercury regulator (Fig. 2, A). The bottom third of the vacuum-tight metal container and its enclosed tissue chamber is immersed in a 3 gallon Dewar flask containing a bath of normal butyl-alcohol and solid CO<sub>2</sub>. Under most conditions the temperature of the bath is in the region of  $-78^{\circ}\text{C}$ .

The principles of operation of the device are as follows: If the current in the heating coil were permanently left off, the copper cylinder containing the tissue would reach an equilibrium temperature such that the heat conducted down the steel support tube to the cylinder just equalled that lost through radiation and conduction through residual gas to the cold ( $-78^{\circ}\text{C}$ .) wall of the casing. On the other hand if the current to the heating coil were left on indefinitely another equilibrium would be reached, of value depending on the foregoing factors plus the power dissipated in the coil; in our present outfit it is around  $0^{\circ}\text{C}$ . Thus, if the mercury regulator (activated by the expansion and contraction of the alcohol in the chamber) around which is the heating coil, is so arranged that the heating current is on at all cylinder temperatures below an assigned one within the  $0^{\circ}$  to  $-70^{\circ}\text{C}$ . range, and off at all higher ones, the effect will obviously be to hold the tissue-chamber temperature constant within limits set by the sensitivity and thermal lag of the thermostat. By the simple process of adding or removing mercury from the thermostat any desired temperature of dehydration may be had within the limits of  $0^{\circ}$  to  $-70^{\circ}\text{C}$ . Small adjustments are made with a syphon

<sup>16</sup> Gersh, I. *Anat. Rec.*, 53: 309, 1932.

<sup>17</sup> Scott, G. H., and Williams, P. S. *Anat. Rec.*, 66, 1932.

tube (Fig. 2, D) set in the alcohol system attached to the mercury regulator.

Fortunately, the apparatus is so arranged that it can be operated by a person inexperienced in the handling of physical equipment. Its sole requirements for consistent operation over a long period of time is that solid  $\text{CO}_2$  be available and that the tapered joint be made vacuum tight with stopcock grease. Other operation is reduced to the manipulation of the stopcocks and of two electric switches (Fig. 2, E and G). Naturally, it is adaptable to any sort of low-temperature dehydration procedure.

The method of dehydrating tissues at  $-32.5^\circ\text{C}$ . practiced routinely in this laboratory is as follows:

(1) Seventy-five to 100 c.c. of iso-pentane (practical) is placed in a pyrex tube and lowered into a liquid-air container. When, after frequent stirrings of the iso-pentane, the liquid air ceases to boil vigorously it may be assumed that a temperature equilibrium has been reached (about  $-190^\circ\text{C}$ .).

(2) Tissues are removed from the animal (under anesthesia) and plunged directly into the cold iso-pentane. Within a few seconds they are completely frozen and are ready for placement in the tissue container of the cryostat.

(3) Tissues are removed from the iso-pentane and hurriedly dried with a towel and placed in the cryostat which has been allowed to cool to  $-70^\circ\text{C}$ . previously. *Speed in handling tissues at this step is important. Under no circumstances should the tissues be allowed to melt or even warm appreciably.*

(4) The switch (Fig. 2, E) controlling the heating element of the cryostat is turned on for a few minutes at a time and over a period of thirty minutes the temperature of the tissue container raised to  $-32.5^\circ\text{C}$ . This temperature is attained when the mercury of the regulator has reached its proper location (Fig. 2, F) in the thermostat.

(5) The vacuum pump is started (Fig. 2, G) and the heating element switch (Fig. 2, E) set at the "on" position.

(6) Tissues remain in the cryostat for the period of time necessary to dehydrate them thoroughly. The time factor is one which must be determined by experiment as some tissues take longer to dehydrate than others. Seventy-two hours generally suffice.

(7) The vacuum is broken and the tissues removed and plunged immediately into a bath of melted paraffin. Slight vacuum is applied until the tissues sink into the paraffin. It can be assumed that they are thoroughly infiltrated when this happens.

(8) The tissue blocks are then imbedded and prepared for cutting in the routine fashion.

(9) Sections are placed on clean, dust-free slides and pressed down with the ball of the thumb. No fixative is necessary to make them adhere to the slide.

(10) Incinerations are then preformed in the routine manner.

**2. Preparation and Incineration of Tissues.** The initial procedure recommended by Policard involved the use of free-hand sections

of unfixed tissue or paraffin sections. Later the advantages of using frozen sections were pointed out (Policard and Okkels<sup>18</sup>) and Schultz-Brauns<sup>19</sup> developed a satisfactory and ingenious technique for obtaining relatively thin ones of unfixed tissue. These sections are particularly suitable for incineration, but are too thick for use in cytological studies, especially with the incineration method. Since the supporting tissue is ignited, the skeletal residue of the cell is fragile. Thick sections of tissue collapse when incinerated and a false impression is given of the amount of ash present as the remains of perhaps ten cells may occupy the space of one. This renders the interpretation of even gross histologic findings difficult.

Then, too, the use of the cold knife as advocated in Schultz-Brauns' method is conducive to the collecting of moisture from the air on the tissue section. This at first thought might seem to be a purely theoretical objection, but when one stops to consider that even the smallest droplet of condensed moisture will cover many cells and, by increasing their water content, bring about a diffusion of salts of some magnitude, it becomes a technical error within the realm of observation. But this objection is a small one when one remembers that sections so prepared must be dried at room temperatures or over a Bunsen burner before they are incinerated. Such a procedure, even with fixed tissues, is capable of introducing error which cannot be overlooked (Scott<sup>20</sup>).

The most satisfactory cytologic details are obtained by using paraffin sections. Thin pieces of tissue are fixed for twenty-four hours in a mixture of 9 parts of absolute alcohol and 1 part of neutral formalin. Dehydration is completed by several changes of absolute alcohol and the tissue is passed into xylol.

After the tissue has been completely cleared, it is imbedded in paraffin in the usual way. Sections from 3 to 5 $\mu$  in thickness are cut and mounted on ordinary glass slides of good quality. No fixative is required to make them adhere to the glass. Water is to be altogether avoided; not because it will dissolve salts from a slice of tissue which has been infiltrated with paraffin, but for the reason that small droplets of it often remain beneath the section and later when the paraffin is melted these enter the tissue and cause a disarrangement of the soluble mineral salts. In place of water a small drop of absolute alcohol or liquid petrolatum may be used to bring about flattening (Policard and Okkels<sup>21</sup>), although in most cases nothing is required if the sections are handled carefully.

It is necessary throughout the procedure to avoid contamination with

<sup>18</sup> Policard, A., and Okkels, H. *Anat. Rec.*, 44: 349, 1930.

<sup>19</sup> Schultz-Brauns, O. *Ztschr. f. wiss. Mikr.*, 48: 161, 1931.

<sup>20</sup> Scott, G. H. *Proc. Soc. Exper. Biol. & Med.*, 32: 1428, 1935.

<sup>21</sup> Policard, A., and Okkels, H. *Anat. Rec.*, 44: 349, 1930.

dust. This means that the paraffin must be absolutely clean, the xylol carefully filtered, the slides washed in distilled water several times, partially dried with alcohol and wiped with a dust-free cloth. Slides thus

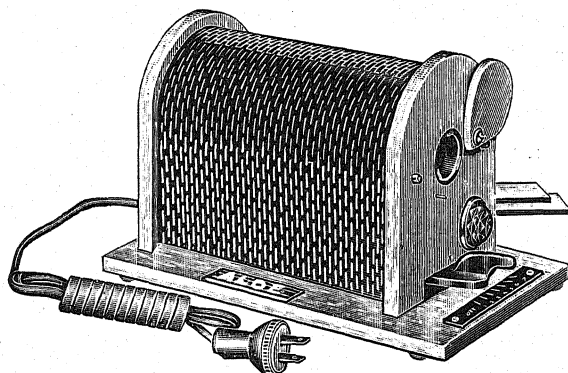


FIG. 3. Small quartz tube electric furnace for incineration.

prepared should be stored in some sort of container which does not admit dust. After the paraffin sections are mounted on the slide the necessity for guarding against dust contamination is doubly important.

It is usually helpful to make serial sections. Alternate ones are mounted for incineration and the remaining sections fixed to slides with egg albumin and colored with any of the usual histologic stains. These latter serve as controls and permit the exact localization of any portion of the tissue when compared with the incinerated preparations. The sections for incineration, if mounted with absolute alcohol are allowed to dry, and if mounted with liquid petrolatum are drained, and then placed in a special quartz tube oven (see Fig. 3) similar to that described by Policard and Okkels.<sup>22</sup> Policard recommends burning for about fifteen minutes, the final temperature being from 500° to 600°C. Better results have been obtained by lengthening the incineration to thirty-five minutes; ten minutes of this time being taken in raising the temperature the first 200 degrees. The balance of the time (twenty-five minutes) is used in attaining the final temperature of about 650°C. This arbitrary period set for incineration should be varied to meet the demands of different tissues since some ash quite readily while others can be completely ignited only with the greatest difficulty. Special attention should be given to the first rise in temperature of 100°. Most of the shrinkage in ordinary tissues, particularly those containing many elastic and collagenic fibers, takes place between 60° and 70°C. If this range in temperature

<sup>22</sup> *Op. cit.*

is passed through hurriedly, shrinkage is great for all cells, but if taken slowly it is confined largely to the connective-tissue elements. Policard and Ravault<sup>23</sup> have studied this phenomenon and have succeeded in devising a method whereby it can be largely avoided. After fixation the tissues are placed in absolute alcohol which is brought slowly to the boiling point. This produces practically all the shrinkage which will occur while the tissue is en bloc. The method is especially valuable in the examination of tissues such as blood vessels which are extremely rich in elastic and fibrous tissue. Because of the danger of dissolving salts in considerable quantity boiling of tissue is not advisable when the observer is interested in individual cells.

Convection currents in the air set up by heating the quartz tube are quite sufficient to provide ample oxygen for the ignition and yet, under ordinary circumstances, are not great enough to disturb the ash residue. Schultz-Brauns<sup>24</sup> recommended the passage of a stream of nitrogen through the tube, while Tschopp<sup>25</sup> has made a similar use of oxygen. These methods work very well and in general permit the use of lower temperatures for incineration, thereby causing the retention of a greater proportion of chlorides in the ash. Policard mentions that sections incinerated in nitrogen, along with a small percentage of oxygen, show less tendency toward the formation of resistant tarry products and consequently oxidize more completely.

In cases where relatively large numbers of slides are to be incinerated the ordinary muffle furnaces are satisfactory to a certain degree. Care must be taken to cover the preparations to protect them from extraneous substances from the furnace walls and roof.

In our laboratory a special quartz tube furnace (see Fig. 4) has been devised which permits exact control throughout the incineration.

The apparatus consists of a 24 inch quartz tube (Fig. 4, A) wound with three separate 600 watt heating elements each of which is controlled by a 44 ohm 3.2 ampere rheostat. The temperatures and lengths of the heating elements are so adjusted that slides being fed through on quartz slabs (Fig. 4, E), moving at a predetermined uniform rate of speed, are exposed to the same gradual gradations of heat experienced in the smaller furnace. The quartz slabs are kept in motion by an electric motor (Fig. 4, B) acting through a set of worm gears (Fig. 4, C) to push them. Long racks (Fig. 4, D, D) extend outward from each end of the furnace to provide room for loading and removing the slides from the 8 inch quartz carriers.

<sup>23</sup> Policard, A., and Ravault, P. P. *Bull. d' histol. appliq. à la physiol.*, 4: 170, 1927.

<sup>24</sup> Schultz-Brauns, O. *Virchow's Arch.*, 273: 1, 1929.

<sup>25</sup> Tschopp, E. W. In: von Mollendorff: *Handb. d. mikrosk. Anat. d. Menschen*, 1: 569, 1929.

With this apparatus the incinerations are absolutely uniform day after day. The only possible way to change the incineration is to alter the rheostat settings and there is seldom an occasion for doing so.

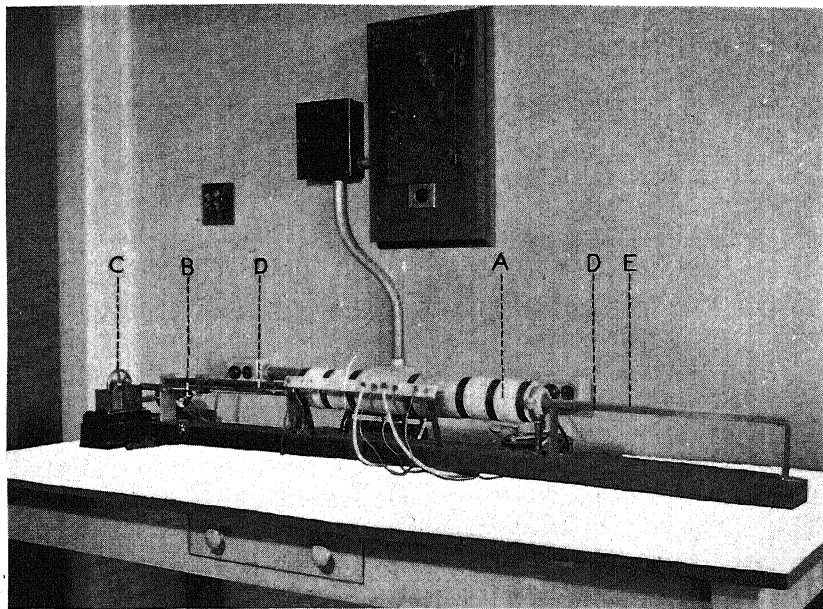


FIG. 4. Large quartz tube electric furnace with automatic control of incinerations. A, Quartz tube wound with heating elements and covered by asbestos insulation. B, Driving motor. C, Speed reduction worm gearing. D, E, Racks for inserting and receiving quartz plates bearing slides.

The routine process for incinerations with the small quartz furnace (Fig. 3) is as follows:

- (1) First ten minutes heat to  $200^{\circ}\text{C}$ .
- (2) Five minutes' gradual heating up to  $280^{\circ}\text{C}$ .
- (3) Five minutes' gradual heating up to  $385^{\circ}\text{C}$ .
- (4) Five minutes' gradual heating up to  $480^{\circ}\text{C}$ .
- (5) Five minutes' gradual heating up to  $580^{\circ}\text{C}$ .
- (6) Three to five minutes' gradual heating up to  $650^{\circ}\text{C}$ .
- (7) Shut off furnace and allow slides to cool in situ for five to ten minutes.
- (8) Remove slides from furnace and lay coverglass over section as soon as slide can be handled with comfort.
- (9) Seal cover glass with wax mixture (see below).
- (10) Examine by dark field.

After incineration the slides are allowed to cool slowly. A coverslip is



placed over the ash and its edges are sealed with one part paraffin, one part beeswax and one part resin by weight. The sealed coverglass serves as a purely mechanical protection permitting the use of oil-immersion objectives, excluding dust and preventing excessive absorption of moisture and efflorescence of the ash. Breathing, even lightly, upon the preparation is to be avoided as it causes a disarray of the ash residue.

It has been suggested that the ash be mounted in Canada balsam. In order that this may be successful, the temperature of incineration must be high enough to cause a fusion of the small particles of ash with the glass in order that they may not be disarranged by the flow of this rather viscous fluid. This technique has been tried many times with but mediocre results. The balsam entirely changes the refractile properties of ash and, despite great care, in the incineration and in the addition of the balsam, there occurs a certain amount of disturbance of the histologic picture. Perhaps a greater objection is that the sections are no longer utilizable for microchemical procedures. A similar disarray of the ash occurs when the sections are dipped in collodion, as suggested by Policard and Okkels,<sup>26</sup> preliminary to the application of various reagents to determine the nature of the ash. The disturbance of optical properties is a serious drawback with this particular technique, for a section having abundant ash before treatment with collodion frequently appears to have lost most of it. Perhaps the ash deposits remain but no longer refract light in their characteristic manner.

**3. Methods of Observation.** Policard<sup>27</sup> first suggested oblique illumination at an angle of approximately  $30^\circ$ . He did so because only a very small amount of material is present and most of it is transparent so that direct illumination reveals very little in properly incinerated preparations. Because of the extremely high temperature of the ash and its subsequent slow cooling, a certain amount of water is taken up from the air, probably changing the structure of the residue. The tiny mass of ash appears to be a fusion of minerals arranged in small hemispherical heaps with the flat side next the glass slide. Light striking the flat side of the hemispheres is passed through, with varying degrees of diffraction; while that striking the small masses from an angle is reflected from the surface. In Policard's more recent publications he advises the use of a parabolic mirror fastened to the objective in such a way that the beam of reflected light can be directed at will. These methods of illumination are quite satisfactory and even desirable for low-power observations, but because of the mechanical difficulties which arise, they are not practical for use with oil-immersion objectives. The cardioid condenser manufac-

<sup>26</sup> Policard, A., and Okkels, H. *Anat. Rec.*, 44: 349, 1930.

<sup>27</sup> Policard, A. *Bull. Soc. Chim. de France*, 33: 1551, 1923.

tured by Zeiss provides an incident source of light which passes through the slide and then strikes the object at an acute angle. Strong light which comes from below is, for both technical and optical reasons, better adapted for high-power objectives.

It is important to have a proper source of light when the cardioid condenser is used. The Zeiss point-o-light lamp is satisfactory when conditions are carefully controlled. It requires exact centering of the condenser and skillful adjustment of the reflecting mirror. The use of a carbon arc lamp in viewing ashed preparations seems to produce an excessive amount of longitudinal aberration. The large Spencer illuminator fitted with a 500 watt projection type bulb has proved to be a satisfactory source of light both for ordinary observation and for photography. The daylight filter or a ground glass renders observation more restful and sometimes more exact, but frequently obscures minute particles of ash revealed with the unscreened light. Mercury vapor lamps yield light which gives excellent differentiation of individual particles but makes the recognition of colors difficult.

It is useful to have two identical microscopes connected with a comparing ocular. One instrument is provided with an ordinary bright-field condenser and the other with a dark-field illuminator. With this arrangement the control stained preparation can be viewed in the same ocular with the incinerated section. This is a necessity when carefully controlled comparisons of stained tissues and incinerated preparations are being made. It is also helpful when the observer is attempting to locate the same structure in both types of sections. After some experience is gained in the observation of incinerations the use of two microscopes is superfluous as most cells and tissues can be readily recognized from the appearance of their mineral deposits.

**4. Appearance and Interpretation of Incinerated Sections.** A section of tissue which has been properly incinerated should show no organic material. In the ash should be all of the mineral components, with the possible exception of a part of the phosphorus and sulphur, not volatilized by heat of 650°C. Furthermore, these elements should be in the same topographic relationship as in the fixed tissue. Whether or not this represents the exact condition in living cells is, of course, open to question. There is some evidence that it does; for photographs of living cells taken by means of ultraviolet light of wave length 2750Å show absorption of these rays by areas which on incineration reveal large deposits of inorganic salts (Scott<sup>28</sup>). In this connection one must take into account the possibility of certain substances, perhaps even pre-formed organelles of the cell, acting as concentrating surfaces for the

<sup>28</sup> Scott, G. H. *Science*, 76: 148, 1932.

inorganic salts during the process of fixation. Then, too, the uniform distribution of the ash, particularly throughout the cytoplasm, suggests that the inorganic salts were dispersed evenly in the living cell, but that on fixation many small foci of crystallization came into being. The use of ultraviolet photography, giving especial attention to the use of different wave lengths, along with microincineration, might yield answers to some of these questions. A comparison of the pictures obtained from living cells by ultraviolet light, polarized light and dark-field illumination with similar tissues after incineration, as has been made by Gage, Day and Starrett,<sup>29</sup> may give information as to the chemical structure of protoplasm which has hitherto been lacking.

The recognition of individual elements in incinerated sections is difficult. Calcium is present in the form of calcium oxide; magnesium in the form of magnesium oxide; iron as ferric oxide and silica as a silico-calcareous compound. Indeed, all the elements common to tissues remain if the heat of incineration has not been great enough to volatilize them. Some phosphorus is lost by the combination of phosphates with the silica in the glass slide to produce silicates and phosphorus pentoxide which is reduced when heated in the presence of carbon. The problem of the optical differentiation of some of these salts is indeed a trying one and at present impossible of solution.

(a) *Iron*. Fortunately, iron when oxidized possesses a more or less intense and individual coloration. This color varies from yellow to deep red, and, according to Policard,<sup>30</sup> its depth is conditioned by the amount of iron present. He has gone even further in this interpretation of iron oxide and stated that perhaps the yellow to brown colored ash is derived from organically bound iron and that the deep red indicates free iron. But in dark-field illumination any carbon remaining in the preparations also gives a yellowish to red color so that caution is necessary. Carbon appears black with transmitted light. Marza, Marza and Chiosa<sup>31</sup> made a careful comparison by four different methods designed for the demonstration of iron and found microincineration to yield excellent results.

When examining sections for iron it is well to be extremely careful with the microtome knife. A freshly sharpened blade will give rise to profuse deposits of iron throughout the tissue. These artifacts can usually be recognized because of their orderly arrangement. After a knife

<sup>29</sup> Gage, S. H., Day, M. G., and Starrett, C. C. *Anat. Rec.*, 55: 4 suppl., 17, 1933.

<sup>30</sup> Policard, A. *Compt. rend. Acad. d. sc.*, 176: 1187, 1923.

<sup>31</sup> Marza, V. D., Marza, E., and Chiosa, L. *Bull. d' histol. appliq. à la physiol.*, 9: 213, 1932.

has been used for cutting 40 or 50 sections the small particles of iron left in the tissues are almost negligible.

(b) *Silica*. This is another element which can be recognized with certainty for it retains its crystalline structure and after heating, when viewed with polarized light, it is doubly refractive. It must be remembered, however, that silica is in all probability present as a silico-calcareous compound, giving the false impression that the ash contains more silica than it actually does. Moreover, when certain of the compounds present in tissues are fused on a glass surface there is a combination of the silica in the slide with the tissue residue. This unfortunate but unavoidable condition introduces another complication in the interpretation of results.

(c) *Calcium*. Calcium can be detected with the aid of the simple yet fairly sensitive gypsum reaction although Moreau<sup>32</sup> cautions against too literal interpretation of the results secured. To perform this test the salt is dissolved in a "microdrop" of tenth-normal hydrochloric acid; later a "microdrop" of tenth-normal sulphuric acid is added to cause the formation of the typical needle-shaped crystals of calcium sulphate ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) (See Ku's<sup>33</sup> Fig. 12). The recognition of magnesium in the presence of calcium is not feasible unless the tissue is subjected to spectrographic analysis (Policard, Morel and Ravault<sup>34</sup>).

Schultz-Brauns<sup>35</sup> offers a method for the detection of calcium in ashed preparations which is to some degree successful. The incinerated sections are carefully immersed in distilled water for two or three minutes and then removed and dried. Such treatment is said to remove the more soluble salts, potassium, sodium and perhaps others as well, and leave the insoluble ones, among these magnesium, manganese, calcium, and so on. But calcium oxide is more soluble in water than the oxides of other elements found in animal tissues. For this reason, the ashes remaining in situ after the water treatment are probably more complex than was at first supposed. Furthermore, in the complex fusion mass, resulting from the heating of the elements and their compounds present in tissues, there are many possibilities of chemical combinations.

(d) *Sodium and Potassium*. Policard and Pillet<sup>36</sup> have suggested a method, said by them to be generally useful in the incineration technique, whereby sodium and potassium may be retained in tissues. They state that sodium and potassium are probably present as chlorides in

<sup>32</sup> Moreau, P. *Bull. d'histol. appliq. à la physiol.*, 8: 245, 1931.

<sup>33</sup> Ku, D. Y. *Am. J. Path.*, 9: 23, 1933.

<sup>34</sup> Policard, A., Morel, A., and Ravault, P. *Compt. rend. Acad. d. sc.*, 194: 201, 1932.

<sup>35</sup> Schultz-Brauns, O. *Virchows Arch.*, 273: 1, 1929.

<sup>36</sup> Policard, A., and Pillet, D. *Bull. d'histol. appliq. à la physiol.*, 3: 230, 1926.

the tissues and that their conversion into sulphates can be accomplished by exposing sections to the fumes of sulphuric anhydride. The sulphates are much more stable compounds and resist the heat of incineration.

(e) *Lead*. Tada<sup>37</sup> and Okkels<sup>38</sup> have been able to differentiate lead in the tissues of experimental animals by exposing the ashed section to hydrogen sulphide. Lead sulphide is formed and shows up as black granulations. There are also other sulphides, for example, that of iron, which might be formed and which have practically the same optical qualities. These sulphides are also difficult to distinguish from carbon deposits in imperfectly incinerated preparations.

(f) *Uranium*. Policard and Okkels<sup>39</sup> mention the possible detection of uranium salts in incinerated sections of tissue by means of their well-known fluorescent properties when exposed to ultraviolet light. This has been tried with success when large amounts of salts were present. The color of fluorescence of chemical compounds is apparently in some measure specific; but it is believed that the presence of exceedingly small amounts of impurities gives the fluorescent color not of the compound in question but of the impurity. The use of ultraviolet is but one means of exciting fluorescence, since many salts react in a similar, although perhaps more specific manner, to x-rays and to radium emanations, particularly alpha rays.

The general appearance of the ash is not always the same and without doubt these variations on further study will reveal chemical differences which have thus far eluded us. In some tissues the ash is decidedly granular and grayish white in color; more frequently, it is a brilliant white and chalky in appearance. At times the minerals are homogeneously dispersed and grayish white; in other preparations the ash residue has a distinct bluish-white tint. The minerals may even be sky blue in certain tissues particularly in the epithelia of the skin and digestive tract. Mason<sup>40</sup> has introduced evidence that the blue coloration of microscopic objects may be due merely to the size and state of dispersion of the substance. Whether these findings are applicable to dark-field observations remains to be determined. No explanation for these differences in appearance can be offered at present. The ash is rarely crystalline. It is possible that the refractive properties of the residue will serve to partially reveal its identity.

<sup>37</sup> Tada, K. *Verhandl. d. Japan. path. Gesellsch.*, 16: 128, 1926.

<sup>38</sup> Okkels, H. *Bull. d'histol. appliq. à la physiol.*, 4: 134, 1927.

<sup>39</sup> Policard, A., and Okkels, H. *Handb. d. biol. Arbeitsmethoden* (Abderhalden), 5: 1815, 1931.

<sup>40</sup> Mason, C. W. *J. Physical Chem.*, 35: 73, 1931.

### III. Quantitative Measurement of Mineral Residue of Tissue Sections

One of the things to be desired in the study of microincinerated sections is an accurate means of estimating the relative quantities of inorganic salts in various cells. Such a method would permit a quantitative comparison of tissues taken from animals subjected to experimental procedures with similar normal cells. Furthermore, it should make it possible to study such processes as normal growth and differentiation in a more exact manner than is possible with only qualitative estimates. A quantitative method has also to commend it the fact that pathologic and comparable normal tissues can be examined with more exactitude.

The accurate quantitative measurement of ash in different tissues is obviously a difficult problem, but rough estimates of comparative amounts can be made as with any type of microscopic preparation. The former demands the use of physical apparatus not commonly found in laboratories of histology. A standard method of photography employing standardized development of the exposed plates has been suggested by Schultz-Brauns.<sup>41</sup> But it is practically impossible to control and repeat conditions exactly, especially with an arc lamp as a source of light. Since the cardioid condenser is an instrument primarily designed for accurate point focusing of a light beam and even the inexperienced observer is aware of an improper adjustment, it therefore becomes the instrument of choice for quantitative estimation of the ash residue.

When the thickness of the section is  $4\mu$  the residue after incineration is practically a single layer for there is but little evidence of piling-up of mineral. When the ash particles are more numerous, yet individually of about the same magnitude, as they usually are, more light will be reflected into the eye of the observer. If the mineral is in a single layer on the slide and is so disposed that its compactness offers but little opportunity for absorption of the incoming light rays, then it is probable, if the salt deposits are of approximately the same nature, that the amount of light reflected from their exposed surfaces will be roughly proportional to their quantity. With these assumptions as a basis, a means has been developed for measuring the intensity of the light reflected from the minerals remaining after incineration (Scott<sup>42</sup>). The method involves the use of a gas-filled photoelectric cell and a suitable amplifying

<sup>41</sup> Schultz-Brauns, O. *Ztschr. f. wiss. Mikr.*, 48: 161, 1931.

<sup>42</sup> Scott, G. H. *Proc. Soc. Exper. Biol. & Med.*, 30: 1304, 1933.

circuit for the current set up by the cell when light from the microscope strikes it (Williams and Scott<sup>43</sup>).

The apparatus consists essentially of a permanently mounted micro-

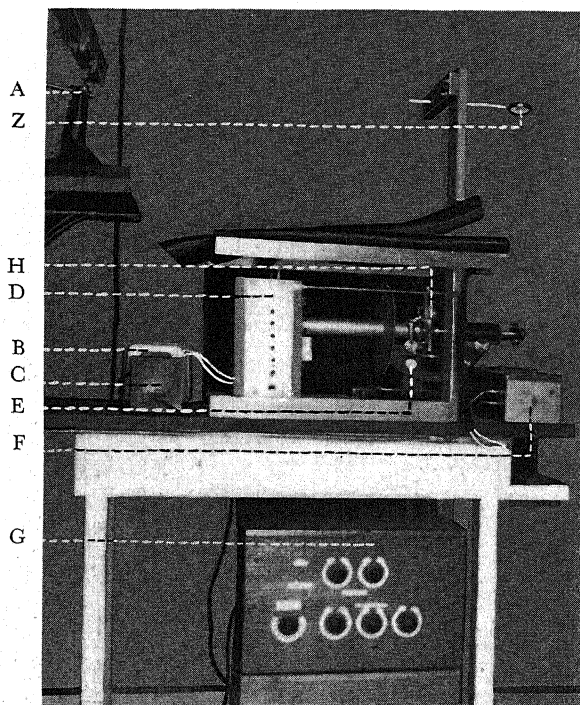


FIG. 5. Dark-field photometer apparatus. A, Lamp and scale of wall galvanometer. B, Constant voltage regulator. C, Step-down transformer. D, Ventilated lamp housing. E, Microscope. F, Photo-cell box. G, Amplifier and battery cabinet with control dials. H, Mechanical stage capable of three-way movement. I, Reflecting mirror.

projection system, the images from which are focused on a dark-field photo-cell box. The whole assembly, with the photo-cell box in position, is shown in Fig. 5, set up in a small darkroom.

The lamp and scale (Fig. 5, A) for the Leeds and Northrup type R galvanometer is shown on the shelf to the left. On the table, from left to right: A constant voltage regulator (Fig. 5, B) and a step-down transformer (Fig. 5, C); the ventilated wooden box housing the 6 volt, 108 watt ribbon filament projection lamp (Fig. 5, D); the microscope assembly (Fig. 5, E); the photo-cell box (Fig. 5, F), the amplifier cabinet (Fig. 5, G) for which is under the table.

<sup>43</sup> Williams, P. S., and Scott, G. H. *J. Opt. Soc. Amer.*, 25: 347, 1935.

The microscope has a Zeiss aplanatic 1.2 condenser, a Leitz number 3 objective, various oculars, and a clamped-on 90° reflecting prism.

A fixed diaphragm composed of a disk slightly larger than the aperture of

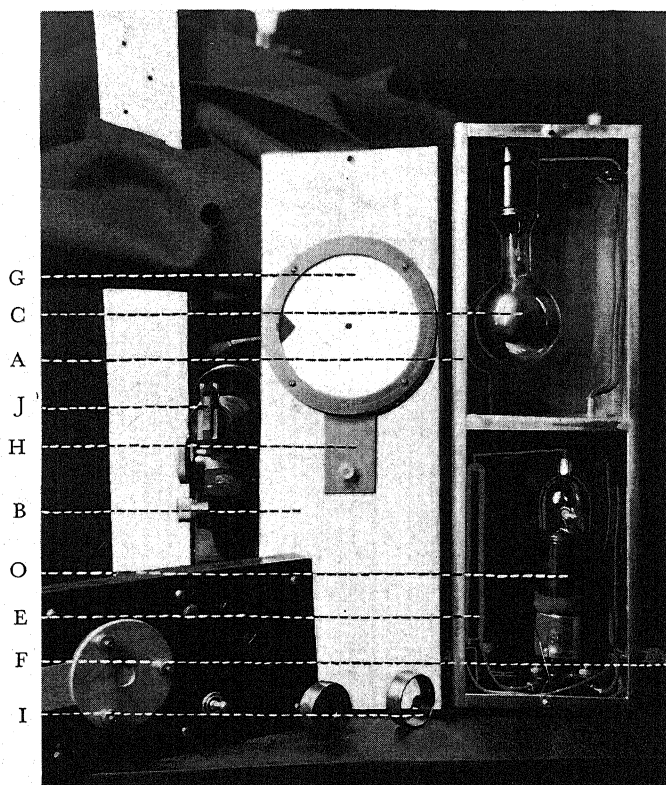


FIG. 6. Photo-cell box of dark-field photometer. A, Copper-walled box. B, Lid for box. C, Photoelectric cell. D, FP54 Photron. E, High-resistance shunt. F, Cable to galvanometer and to amplifier box. G, Cardboard slit arrangement to admit light to photo-cell. H, Shutter for slit. I, Dark-field ring for substage condenser. J, Prism on microscope ocular.

the objective (Fig. 6, I) is fitted into the ring of the condenser lens. The mechanical stage (Fig. 5, H) for the slide gives rack-and-pinion adjustments laterally and vertically and a fine screw adjustment axially; the latter feature had to be built specially. The whole arrangement is carried by the microscope frame, the stage having been removed to increase clearance.

Details of the photo-cell box are shown in Figure 6.

At the right is the cell box with the cover (Fig. 6, B) standing beside it. The box (Fig. 6, A) of heavy copper, is light-tight and gives good electrostatic screening. The photoelectric cell (Fig. 6, C) is directly connected to an FP54 Photron



(Fig. 6, D); the  $10^9$  ohm high-resistance shunt (Fig. 6, E) is in the shadow. The connections to the DuBridge and Brown amplifier<sup>44</sup> (using 12 volts of lead cells), photo-cell B battery, etc., run to the cabinet shown in Figure 5, through a flexible

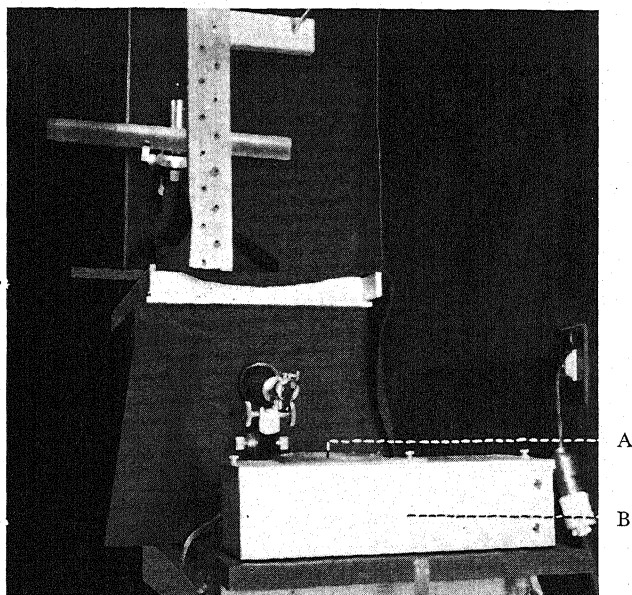


FIG. 7. Photometer set up for operation. A, Slit in photo-cell box. B, Photo-cell box containing photoelectric cell and amplifying tube.

shielded cable (Fig. 6, F) which also brings the galvanometer leads back to phone sockets on the box. The cover of the box carries rotatable and interchangeable  $\frac{3}{8}$  inch white cardboard disks serving as slits (Fig. 6, C); any size and shape aperture falling within a  $\frac{3}{4}$  inch circle may be used. A sliding shutter (Fig. 6, H) works under these to permit exposure of the photo-cell at the time desired.

The apparatus assembled for use is shown in Figure 7.

To use the apparatus the slide is inserted into the mechanical stage and the barrel of the microscope adjusted to bring the object into clear focus. The reflecting prism (Fig. 6, J) swung into position and the transmitted light reflected upward to the small mirror shown in Fig. 5, I, and from thence down to the cardboard disk (Fig. 7, A) on the lid of the photoelectric cell box (Fig. 7, B). With this arrangement a magnification of 700 is obtained which for most measuring purposes is quite adequate.

The first step in the operation is to focus the filament image of the lamp sharply and centrally on the cardboard screen; this is done with

<sup>44</sup>DuBridge, L. A., and Brown, H. *Rev. Sci. Inst.*, 4: 532, 1933.

the dark-field diaphragm removed and with the ash-bearing glass slide in position. It is convenient to increase the size of the filament image to about 4 inches in width by putting a 12-power convex lens in front of the lamp housing. These adjustments are made but once, then the dark-field diaphragm is inserted and, with all the felt curtains down (as in Fig. 7) to shut off stray light from the lamp housing, etc. and the room almost completely dark, the image of the ash deposits is focused on the screen with the axial adjustment of the stage. The image is faint but sufficiently visible. The portion to be measured is brought close to the center of the screen, using the lateral and vertical stage adjustments; the precise setting of the aperture in the screen is made by moving the photo-cell box itself. The galvanometer deflection when the shutter is pulled, is proportional to the scattered light passing through the aperture. The "background" is not completely dark, so that it is necessary to subtract the small clear slide deflection to get the light scattered by the ash itself. The resulting number is proportional to the amount of ash (if small) on the area taken, and can be compared with a similar measure for another area of the slide without concern as to the actual amounts involved.

With 700 magnification, the 0.1 galvanometer shunt, and a  $\frac{1}{4}$  inch aperture in the screen, about half-scale deflection (25 cm.) is obtained from the ash due to a  $5\mu$  section through a single nucleus of an hepatic cell.

## THE CENTRIFUGE MICROSCOPE

ETHEL BROWNE HARVEY

The centrifuge microscope is an instrument with which one can observe living cells under high magnification while they are actually rotating in a centrifuge. It was designed by E. N. Harvey and A. L. Loomis and is made by the Bausch and Lomb Optical Company (Fig. 1). The instrument consists essentially of a motor, a rotating disk containing part of the optical system of a compound microscope, a stationary eyepiece, and a source of light. There is an opening near the periphery of the upper surface of the rotating disk over which a slide containing material to be observed can be placed. Under this opening, mounted in the rotating disk, is a right-angled prism and the lenses of the microscope objective, so arranged that when under the source of light, an image of the material on the slide is reflected horizontally to the axis of rotation and then vertically upward by another right-angled prism to the stationary eyepiece, as shown in the diagram (Fig. 2). A condenser throws on the material an image of an incandescent filament parallel to the radius of rotation. The slide is under the light for a small fraction of a second during each revolution of the disk, and the series of these very rapidly intermittent images gives a continuous picture just as in the motion pictures. Blurring is prevented by bringing the images to the center of rotation of the centrifuge. The steadiness of the image is indicated by the very clear photographs which can be taken while the machine is rotating 10,000 times a minute (Fig. 5). Excellent motion pictures have also been taken of eggs pulling apart in the machine.

The speed of rotation can be controlled by the speed control box, a combination of rheostat and voltmeter (see Fig. 1), and can reach as much as 10,000 revolutions per minute (radius is 8.6 cm.). The instrument is made with both low (16 mm.) and high (4 mm.) power objectives, with a simple device for changing from one to the other. Any ocular can be used, but a 10 $\times$  ocular is supplied, giving a magnification of 100 $\times$  with the 16 mm. lens and 400 $\times$  with the 4 mm. lens. The slide (Fig. 3) is especially made, consisting essentially of a square depression slide (1 inch square) over half of which is a coverslip fused and annealed to withstand the great centrifugal force. This is placed in the machine upside down so that the coverslip lies directly over the prism and lenses in the rotating disk. The slide is made so that the edge of the depression, to which material is centrifuged, comes directly under the

optical axis of the condenser. It is sometimes advantageous to put a small amount of solution isotonic with the cells, and of slightly greater density (such as sucrose) at the bottom of the slide before inserting the

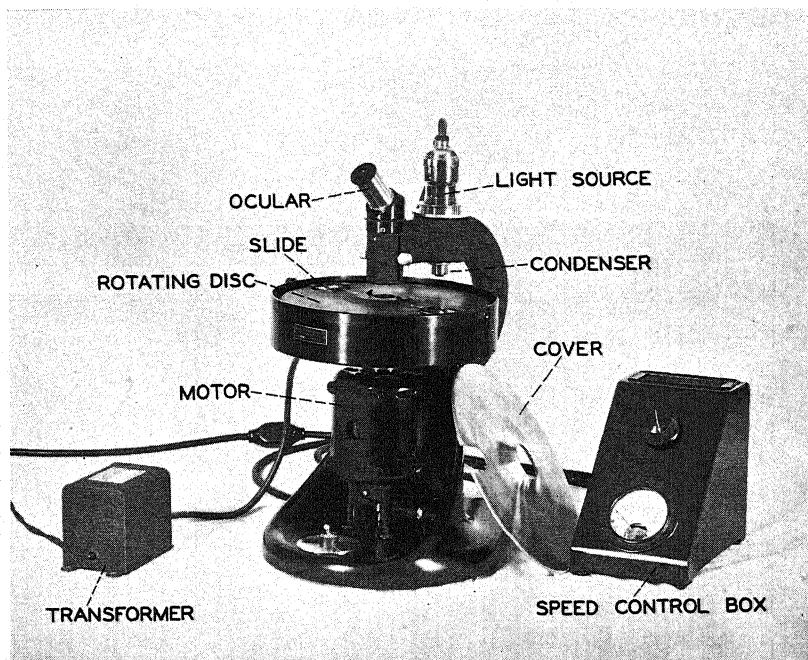


FIG. 1. Centrifuge microscope and its accessories.

material to be studied, to act as a cushion to prevent crushing of the cells.

The centrifuge microscope can be used for the observation of the displacement of granules in living cells such as amebae, paramecia, or the eggs of the sea-urchin or starfish; the heavier granules, such as the pigment in the *Arbacia* egg, are thrown centrifugally, and the lighter granules, such as oil, go centripetally, and thus a definite stratification is formed. Many cells can be observed in the process of breaking into fragments when subjected to centrifugal force (Figs. 4 and 5). Such observations can be used to measure the rate of movement of granules, from which the viscosity can be calculated. Surface forces can also be calculated from observations with the centrifuge microscope. One can observe the effect of centrifugal force on mitotic figures and other nuclear and cytoplasmic structures visible in the living cell. Differences between unfertilized and fertilized eggs can also be studied (Figs. 4 and 5). A more complete account of the instrument is given in an article

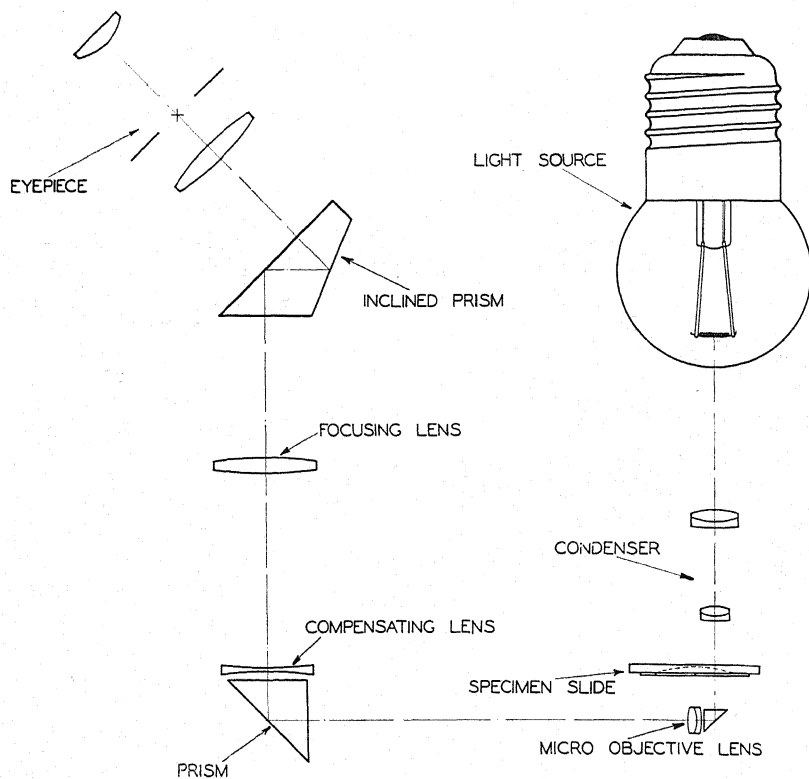


FIG. 2. Diagram of optical system of centrifuge microscope.

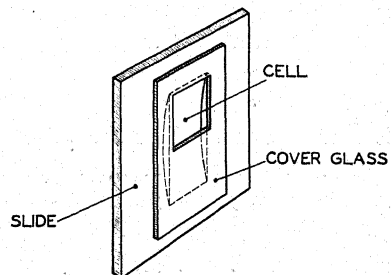


FIG. 3. Specimen slide, 1 inch square, in which material is centrifuged.

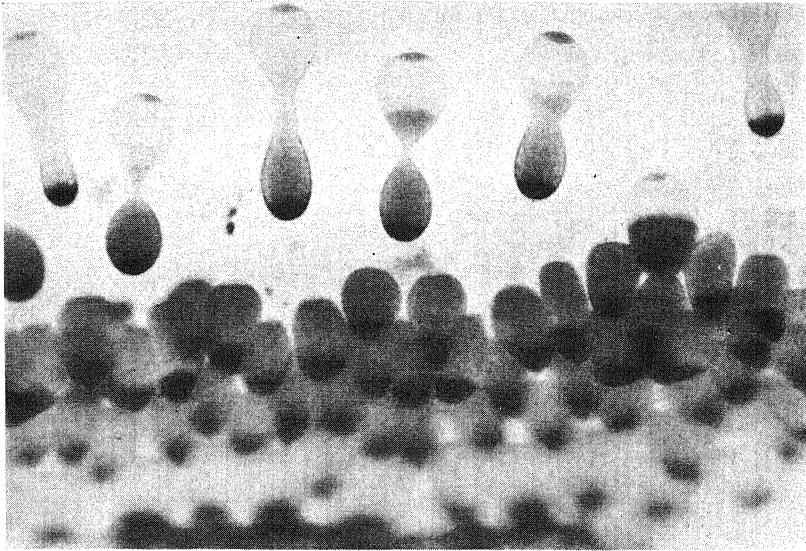


FIG. 4. Photograph of unfertilized eggs of *Arbacia punctulata*, stratifying and pulling apart; taken immediately after removal from centrifuge microscope, while still in slide.

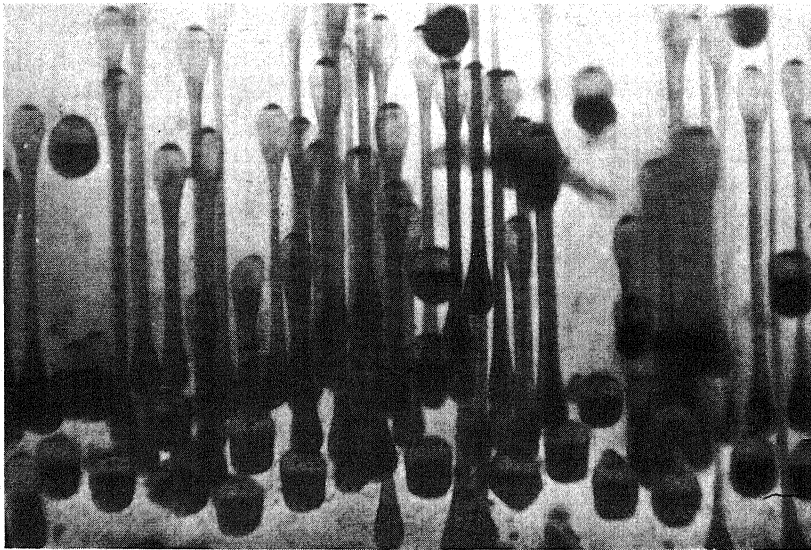


FIG. 5. Photograph of fertilized eggs of *Arbacia punctulata*; taken while rotating in centrifuge microscope at about 10,000 r.p.m.

entitled "The microscope-centrifuge and some of its applications," by E. Newton Harvey.<sup>1</sup> Complete directions for the use of the centrifuge microscope are supplied with each instrument.

A modification of the centrifuge microscope (not made commercially) by which two different materials, e.g., experimental and control, can be observed at the same time, side by side in the same field, has been described by E. N. Harvey.<sup>2</sup>

<sup>1</sup> *J. Franklin Inst.*, 214: 1-23, 1932.

<sup>2</sup> *Science*, 77: 430-431, 1933.

# FLUORESCENT MICROSCOPY

CHARLES J. SUTRO

Apparatus 671. Preparation of tissues 672. Observations 673.

It has been shown that radiation of animal tissues (and also many other substances) by filtered ultraviolet rays will cause an emission from them of secondary or visible rays, a phenomenon known as fluorescence.<sup>1</sup> The filter (nickel-oxide glass) through which the ultraviolet waves are passed excludes not only visible light but also the short ultraviolet rays. However, it permits the passage of ultraviolet waves essential for fluorescence, those measuring from 300 to 400 millimicrons in length. By the use of filtered ultraviolet radiation, it is possible to examine tissues both grossly and microscopically. It is with the latter type of examination that we are here concerned.<sup>2</sup>

**1. Apparatus.** Apparatus consisting of assembled units necessary for the undertaking of fluorescent microscopy can be purchased in the open market. The following set-up, however, can be assembled at a comparatively lower cost:

<sup>1</sup> Danckwortt, P. W. *Lumineszenz-Analyse im filtrierten ultravioletten Licht*, Leipzig, Akademische Verlagsgesellschaft, 1929.

Policard, A. *Compt. rend. Soc. de biol.*, 91: 1423, 1924; *Bull. d'histol. appliq. à la physiol.*, 5: 266, 1928.

Kramer, K. *Virchows Arch.*, 274: 215, 1929.

Korbler, J. *Strahlentherapie*, 41: 510, 1931; 43: 317, 1932.

Sutro, C. J., and Burman, M. S. *Arch. Path.*, 16: 346, 1933.

<sup>2</sup> Kohler. *Ztschr. f. wissenschaft. Mikr.*, 21: 129; 273, 1904.

Walkhoff. *Sitzungsb. d. Gesellsch. f. Morphol. u. Physiol. in München*, 33: 7, 1922; *Verhandl. d. phys.-med. Gesellsch.* 49: 159, 1924.

König. *Ibid.*, 49: 160, 1924.

Bommer, S. *Acta dermat.-venereol.*, 10: 390, 1929.

Hartoch, W. *Ztschr. f. d. ges. exper. Med.*, 79: 538, 1931.

Singer, E. *Science*, 75: 289, 1932.

Thenon, J., and Pirotsky, I. *Rev. Soc. argent. de biol.*, 8: 201, 1932.

Eros, G. *Centralbl. f. allg. Path. u. path. Anat.*, 54: 385, 1932.

Radley, J. A., and Grant, J. *Fluorescence Analysis in Ultra-Violet Light*. N. Y.,

Van Nostrand, 1933.

Bommer, S. *Dermat. Ztschr.*, 67: 319, 1933.

Exner, R. *Psychiat.-neurol. Wchnschr.* 35: 319, 1933; 36: 291, 1934.

Querner, F. *Ztschr. f. mikr.-anat. Forsch.*, 32: 444, 1933.

Baroni, B. *Arch. ital. di dermat., sif.*, 9: 543, 1933.

Haitinger, M., and Hamperl, H. *Ztschr. f. mikr.-anat. Forsch.*, 33: 193, 1933.

Hamperl, H. *Virchows Arch.*, 292: 1, 1934; *Arch. Path.*, 19: 838, 1935.



1. An ordinary microscope.
2. As a source of light, a water-cooled quartz mercury vapor arc lamp (Hanovia Chemical Co.).

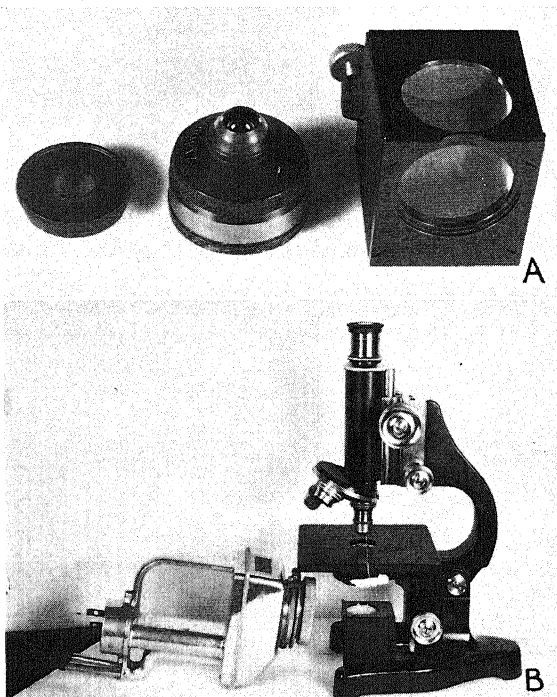


FIG. 1. A. From left to right, special eyepiece filter, quartz condenser and quartz prism reflector. B. Water-cooled quartz mercury vapor arc lamp and parts assembled in microscope. (After Sutro, *Arch. Path.*)

3. A Corex ultraviolet-ray filter (nickel-oxide glass).
4. A quartz prism reflector.
5. A quartz condenser (Abbe type without the iris diaphragm).
6. A special eyepiece cap filter. (Items 4, 5 and 6 from the Bausch and Lomb Optical Co.).

The assembled apparatus is shown in the accompanying figure. All examinations must be done in a completely darkened room.

**2. Preparation of Tissues.** The fresh tissues should be preserved in a diluted solution of formaldehyde U.S.P. (1-10). Fixation in Helly's solution or in other colored fluids interferes with the natural fluorescence. Paraffin sections are prepared from the fixed tissues using any of the ordinary routine dehydrating, clearing and impregnating procedures. Frozen sections may be used. (Celloidin (parlodion) disturbs

the fluorescent effects.) Microtome-cut sections (from 7 to 8 $\mu$  in thickness) are attached by means of egg albumen to special slides which permit the penetration of ultraviolet radiation (these are known as U-V slides, Pfaltz and Bauer). The sections placed on these slides are neither protected with coverslips nor stained. Prior to examination with the fluorescent microscope the sections are immersed in xylene for approximately one-half hour. The sections are then dried in air at room temperature. After such treatment, the attached sections remain still satisfactory for examination even months later.

**3. Observations.** The fluorescent effect depends probably for the most part on the chemical contents of the tissues. This in turn is partly dependent on the condition of the specimen (well preserved or dried) and on the age of the person or animal from whom it was obtained. The color of the fluorescent reaction is not specific, for slight variations may be observed in the same tissues.

Fluorescent microscopy permits the differentiation of some of the constituents of tissue. For example, the examination of an unstained paraffin-embedded section of human spleen reveals purplish fluorescence of the capsule as compared with a deep brown fluorescence of the pulp. The cells of the islands of Langerhans fluoresce reddish as compared with a dark brown tint of the excretory portions of pancreatic tissue. Small foci of pigment were noted by fluorescence in many organs. Some of these were not observed in the stained duplicate sections. Elastic tissue, fat, lipoid and chromaffin material also fluoresce quite distinctly.

Bone fluoresces various shades of blue and white depending, for instance, upon age and upon the factors previously mentioned. (Five per cent solution of nitric acid can be used for decalcification.) Newly deposited bone can be easily differentiated from older bone in the unstained sections.

Fluorescent microscopy is also helpful for the detection of very small amounts of fluorescent dyes in tissue, as for example, eosin, mercurochrome, dibenzanthracene, etc. The sites of deposition of injected porphyrin can also best be determined by fluorescent microscopy.<sup>3</sup> It is interesting that in animals in which ligation of the common bile duct is performed, yellowish fluorescence can be noted in the unstained sections of the nucleus pulposus, in the provisional zone of calcification of growth plates, in the innermost layer of the periosteum, in the blood vessels of fibrocartilage, and of fibrous ligaments, in pulp of teeth, in the odontoblastic and ameloblastic layers, and in the acalcified enamel. The yellowish appearance is absent in the stained duplicate sections. The

<sup>3</sup> Fikentscher, R., Fink, H., and Emminger, E. *Arch. f. path. Anat.*, 287: 764, 1933.  
Emminger, E., and Buchele, B. *Ibid.*, 295: 46, 1935.

yellowish discoloration is apparently directly related to the experimentally produced icterus.<sup>4</sup>

Fluorescent microscopy is also employed advantageously in the photomicrography of the very fine constituents of cells which are not generally perceived by ordinary artificial light.<sup>5</sup>

<sup>4</sup> Sutro, C. J. *Arch. f. Path.*, 22: 109, 1936.

<sup>5</sup> Lucas, F. F. *Science*, 71: 515, 1930; *Proc. Nat. Acad. Sc.*, 16: 599, 1930.

Wyckoff, R. W. G., and Ter Louw, A. L. *J. Exper. Med.*, 54: 106, 1931.

Fowler, E. P., Jr., and Applebaum, E. *Anat. Rec.*, 55: 23, 1932.

Wyckoff, R. W. G., and Ebeling, A. H. *J. Morphol.*, 55: 131, 1933.

Wyckoff, R. W. G. *Cold Spring Harbor Symp. Quant. Biol.*, 2: 39, 1934.

## INDEX

- Absolute-alcohol formalin, 646
- Absorption method for study of teeth by roentgen rays, 382
- Accessories for micromanipulation, 97
- Acetic acid, 553
  - combinations, 556
- Aceto-carmine: Belling's, 165
  - cytological, 264
  - for plants, 165
  - Schneider's, 612
- Acetone, 618
- Acetone-lucidol fixation for blood, 329
- Achucarro's method for centrosomes in nerve cells, 469
- Achucarro's tannin silver method, 508
- Acid alcohol, 619
- Acid Bordeaux, 576
- Acid Congo red, 603
- Acid dyes, 605
- Acid-fast staining, 139
- Acid fuchsin, impregnation with, 349
- Acid green, 590
- Acid green O, 595
- Acid-haemalum, Mayer's, 613
- Acid magenta, 586
- Acid orange, 596
- Acid phloxine GR, 582
- Acid polychrome methylene blue, 122
- Acid Rubin, 586
- Acid yellow R, 576
- Acids, weak, 346
- Acriflavine, 576
- Addison, W. H. F., 437
- Adipose tissue, 628
- Adjective stains, 608
- Adjuvants, 248, 564
  - differential action in fixatives, 564
  - in plant fixing fluids, 207
  - for washing, 11
- Adloff, method of, 393
- Affixing embryos to paper, 284
- Affixing plant sections, 183
- Agar block method, 134
- Agduhr, modification of Bielschowsky's method, 464
- Agitator, 255
- Air-bubbles, 627
  - in damar, 627
  - during spreading, 627
  - in tissue, 628
- Albumen: Mayer's, 620
  - smears for Protozoa, 530
  - water, 22
- Alcohol, 618
  - absolute, 619
  - ammoniated, as a fixative for nerve tissue, 455
  - denatured, 618
  - ethyl, 618
  - for fixation, 533
  - as fixative for plant material, 169
  - as fixative for Protozoa, 530
  - methyl, 619
  - mixtures, 441, 558
- Alcoholic dyes for plant tissues, 186
- Alcoholic-eosin-methylene blue method, for Protozoa, 536
- Alcoholic hematoxylin, 535
- Algae: fixation of, 227
  - fixatives for, 227
  - herbarium specimens of, 226
  - special methods for, 226
  - staining of, 227
- Alizarin blue RBN, 587
- Alizarin carmine, 577
- Alizarin no. 6, 598
- Alizarin red S, 577
- Alizarin red, water soluble, 577
- Alizarin sulphate, 577
- Alizarin-purpurin, 598
- Alkanin, 224
- Allen, Ezra, 246, 279, 552
- Allen's B-15 for chromosomes, 249
- Allen's fluid, 561
  - for plant cells, 216, 217
- Allen's method for mammalian tissues, 249
- Allen's special methods, 249
- Alligation, 625
- Allolobophora, eggs of, 258
  - smearing of, 264
- Altmann's aniline-fuchsin-picric acid method, 266, 268
- Altmann-Gersh technique, 647
- Alum-carmine, 612
  - and picric acid, 612
- Alum-cochineal, 612
  - for embryos, 284
- Alums, 619
- Amanil garnet H, 584
- Amaranth, 577

- Ameba, surface tension of, 104  
 Ameba nucleus, permeability of, 104  
 Amebas: centrifuging, 108  
     granules in, 107  
 Amethyst violet, 577  
 Ammonia carmine, 611  
 Amphibian larvae, Knowler's method of  
     injecting, 57  
 Amplifying circuit of photocell, 665  
 Amyl nitrite, 620  
 Amyloid: Bismarck brown and methyl  
     violet reaction for, 419  
     iodine green reaction, 419  
     Langhans' method for, 418  
     Mayer's stain for, 419  
     methyl violet reaction for, 418  
 Angiosperms, methods for, 243  
 Aniline blue: alc. sol., 600  
     water sol., 577  
 Aniline dyes: formulas for, 610  
     method of application, 604  
     mordanting for, 604  
     solvents of, 575, 603  
     strength of solution, 604  
 Aniline gentian violet, Ehrlick, 137  
 Aniline oil for clearing, 256  
 Aniline red, 586  
 Anthers, fixation of, 213  
 Anticoagulant, non-shrinking, 303  
 Apparatus, 615  
     for micromanipulative technique, 72  
     characteristics of, 73, 74  
     choice of, 70  
 Appleton, J. L. T., 353  
 Aqueous dyes for plant tissues, prepara-  
     tion of, 185  
 Aqueous mounting media for plant tis-  
     sues, 198  
 Arc lamp as source of light, 657  
 Archelline 2B, 578  
 Archoplasmic structures in plant cells, 206  
 Arinkin method for bone marrow, 339  
 Arneith-Schilling count, 122, 126  
 Arsenic acid, for decalcification, 347  
 Artifacts, 628  
 Ash, appearance of, 660  
 Astroscopus raja, 434  
 Astrocytes, 489, 497, 505  
     fibrous, 490  
     protoplasmic, 490  
 Auramin, 578  
     and aniline blue, 189  
 Aurantia, 578  
 Aurin, 600  
 Aurin red, 582  
 Autogenous grafting on ontogenetic ele-  
     ments, 397  
 Avian and mammalian embryos, 250  
 Avian tissues, 250  
 Azidine blue 3B, 602  
 Azidine scarlet R, 603  
 Azo-bordeaux, 579  
 Azo-carmines, 578  
 Azo-carmines, 578  
 Azo rubin, 577  
 Azure, 117  
 Azure A, 578  
 Azure B, 578  
 Azure C, 579  
 Azure I, 593  
 Azure II-eosin, 588  
     and hematoxylin, 328, 339  
 B-3 fixing fluid, 561  
 B-15 fixing fluid, 249  
 B-20 fixing fluid, 249  
 Bacteria: in dairy products, 147  
     hanging drop for, 133  
     isolation of, 105  
     pure cultures, 135  
     slime-forming, Conn's stain for, 146  
     soil, 146  
     stained preparations for, 134  
     staining methods for, 135  
     unstained preparations of, 133  
 Bacteriological methods, 132  
 Balsam infiltration, 202  
 Balsam mounts, cleaning of, 202  
 Balsamic media, 618  
 Barber pipette holder, 66  
 Barrett, method of, 395  
 Bartelmez, G. W., 460  
 Basic aniline dyes, 575  
 Basic fuchsin, 136, 139, 142, 143, 146  
 Basic rubin, 597  
 Basophiles, supravital staining of, 125  
 Basophilia, diffuse, 320  
 Battery fluid, 77  
 Bayberry wax, 258  
 Beers' method for Rhizopods, 546  
 Benda's crystal violet alizarin, 268  
 Bensley's brazilin-wasserblau, 273  
 Bensley's copper-chrome-hematoxylin, 271  
 Bensley's fixative for trophospongium,  
     469  
 Bensley's method for mitochondria, 267  
 Bensley's neutral safranin method, 272  
 Benzamine blue 3B, 602  
 Benzo blue 3B, 602  
 Benzo new blue 2B, 583  
 Benzol, 620

- Benzopurpurin 4B, 579  
 Bergamot oil, for clearing, 251  
 Berlin blue, 577  
 v. Beust method, 374  
 Bichloride of mercury: combinations, 557  
     as fixative, 555  
 Bichromate mixtures, 557  
 Biebrich scarlet, water sol., 579  
 Bielschowsky's ammoniacal silver bath, 409  
 Bielschowsky's silver methods, 435  
     for dental periosteum, 399  
     Foot's modification, 410  
     for gingival innervation, 396  
     for nerve tissue, 435  
     Perdrau's modification, 409  
 Bielschowsky-Maresch method, 407  
 Billingsley, P. R. *See* Ranson, 547  
 Bindschedler's green, 579  
 Birch-Hirschfeld reaction, 419  
 Bismarck brown and light green, 189  
 Bismarck brown R, 579  
 Bismarck brown Y, 579  
 Blackman's alizarin method, 172  
 Blastomeres: Echinoderm, Hörstadius method, 46  
     transplantation of, 46  
 Bleaching methods, 623  
 Bleaching plant sections, 184  
 Blocking and trimming, 17  
     collodion masses, 30  
 Blood: capillaries, microinjection apparatus for, 94  
     cells: vital staining of, 123, 124  
         white, vital staining of, 124, 125  
     citrated, 299, 303, 305, 309, 310, 315, 317  
     collection: citrate method for, 293  
         Osgood method for, 293  
     color index of, 302  
     counting, 287  
         calculations, 291  
         errors, 292  
     drawing specimen of, 287  
     film: special method for, 34  
         dry, 325  
         wet, 331  
     groups: compatibility of, 318  
         method of determining, 315  
         in man, classification of, 314, 315, 316  
     heparinized, 303  
     lancet, 287  
     methods for obtaining, 324  
     oxalated, 297, 303, 305, 309  
     pipette, 287  
     platelets, counting, 293  
     Protozoa, 542, 543  
     Blood—(Continued)  
         samples, use of, 293  
         smears, 34  
         specific gravity of, 306  
         stains, 579  
         supravital staining of, 122  
         technique, 288  
 Bloods, comparing two, 317  
 Body wall, preparation of, 337  
 Boeck's concentration method for Protozoa, 540  
 Boedeker's method for enamel, 372  
 Bone: calcified tissue in, 349  
     celloidin sections of, 350  
     corpuscles, isolation of, 348  
     decalcification of, 346  
     histological technique for, 344  
     marrow: biopsy, 338  
         fixation, 337  
         stains for, 339  
         supravital staining of, 128  
     preparations of, 345  
     sections of, 345  
         without decalcification, 345  
     staining of, 348  
 Borax-carmine, 612  
 Borax-methylene-blue for Protozoa, 543  
 Bordeaux, 577  
 Bordeaux CGN, 584  
 Bordeaux red, 579  
 Bordeaux SF, 577  
 Botanical microtechnique, 155  
 Bouin's fluid, 423, 428, 560  
     modifications of, 560  
     for nerve tissues, 441, 444  
     for plant cells, 217  
     for Protozoa, 533  
 Boxes, slide, 28  
 Brain stem, rapid method for staining, 630  
 Brains, 443, 444  
     effect of fixatives on, 443  
     effect of formalin on, 444  
 Brandt-Rehberg burette, 93  
 Brazil's alcoholic picro-formol-acetic for Protozoa, 534  
 Brazilian, 191, 574  
 Bremer's counterstain after Cox-Golgi method, 449  
 Brilliant blue C, 580  
 Brilliant Congo R, 603  
 Brilliant Congo red R, 603  
 Brilliant cresyl blue, 117, 123, 295, 319, 322, 580  
 Brilliant dianil red R, 603  
 Brilliant fast scarlet B, 601  
 Brilliant green, 580

- Brilliant pink, 599  
 Brilliant Ponceau, 598  
 Brilliant purpurin R, 580  
 Brilliant yellow S, 580  
 Brom chlor phenol blue, 580  
 Brom cresol green, 580  
 Brom cresol purple, 580  
 Brom phenol blue, 581  
 Brom phenol red, 581  
 Brom thymol blue, 581  
 Brown salt R, 582  
 Bryophytes, methods for, 240  
 Buchholz and Blakeslee's method, 244  
 Buckman and Hallisey: counting platelets, 294  
     solution, 322  
 Buds, fixation of, 213  
 Buffalo garnet H, 584  
 Buffer solution, 327  
     for Kingsley's stain, 335  
     for Mommensen's stain, 330  
     for Wright's stain, 327  
 Burket, celloidin-paraffin technique, 366  
 Buttons, terminal, of Cajal, 459  
 Butyl alcohol, 162  
     in paraffin method, 38  
  
 Cabot's rings, 321  
 Caesar red, 584  
 Cajal: fixing bath, 519  
     formalin-ammonium-bromide, 519  
     gold chloride and sublimate, 519  
     method: for astrocytes, 497  
         modification of, 507  
     for dental periosteum, 390  
     of double impregnation (Golgi), 447  
     for neurofibrils, 452  
     modification for calcified tissues, 459  
     for fish brains, 460  
     for frozen sections, 461  
     for large blocks, 460  
     reduced silver methods, 452  
     reinforcer, 520  
     terminal buttons, methods for, 459  
     uranium nitrate silver method, 275  
     uranium-formol method, 467  
 Calcified tissue in bone, 349  
 Calcium, 658  
     microreaction for, 659  
 Calcium salts, in plant tissues, 157  
 Calkins' sublimate alcohol for Protozoa, 533  
 Callose, staining of, 196  
 Canada balsam, 618  
     for plant tissues, 201  
  
 Canalicular apparatus of parietal cells, 118  
 Canary yellow, 578  
 Capillaroscopy in study of teeth, 401  
 Capps volume index method, 304  
 Capsule stain, 145  
 Carbol-xytol-cresote mixture, 520  
 Carbon in sections, 658  
 Carmalum, Mayer's, 612  
 Carmine and cochineal, 573  
 Carmine stains: acid, 612  
     alcoholic, 612  
     alkaline and neutral, 611  
 Carnoy's fluid, 466, 558  
     for plant cells, 214, 216, 217  
 Carnoy-Lebrun's fluid, 558  
 Carothers' method for Orthopteran cells, 251  
 Casares-Gil's flagella stain, 143  
 Castro (De Castro), F., 453, 455, 459  
 Castro's method for calcified tissue, 459  
 Cedar oil for clearing, 251  
 Celestin blue B, 581  
 Cell membrane in muscle, 431  
 Cell organs of contraction, 425  
 Celloidin: for electric organs, 434  
     for teeth, 362  
     technique of Burket, 366  
 Cells in body fluids, supravital staining of, 128  
 Cellular physiology, micrurgical technique in, 101  
 Cellular tissues, subcooling technique, 104  
 Cellulose: sectioning, 157  
     staining of, 195  
 Cements, 617  
     for sealing aqueous mounts, 199  
 Centrioles in nerve cells, 469  
 Cerasin, 579  
 Cerasin red, 600  
 Cerebellum, reduced silver methods for, 453  
 Cerebral cortex, 444  
 Cerebrospinal fluid, supravital staining of, 128  
 Cerebrum, reduced silver methods for, 453  
 Chambers, Robert, 62  
 Chambers' injection apparatus, 87  
 Chambers' micromanipulator, 68  
 Chambers' special methods of microinjection, 54  
 Champy's fluid, 560  
 Champy's bichromate-chromic-osmic mixture, 423  
 Champy-Kull's method, 269, 578  
 Changing fluids, automatic device for, 570

- Charophyceae, preparation of, 229  
 Charter-White's method for interglobular spaces, 387  
 Chase method for sections of teeth, 359  
 Chemical agents for fixation, 552  
 Chick embryos, 280, 282  
   injecting, 52  
 Chicken testes, 250  
 China blue, 577  
 Chlor cresol green, 581  
 Chlor phenol red, 581  
 Chlor-zinc-iodine for cellulose, 195  
 Chloral hydrate as fixative, 455, 458, 459  
 Chlorazol blue 3B, 602  
 Chloroform as fixative, 553  
 Chlorophyceae: fixation of, 228  
   filamentous, fixation of, 229  
   massive, fixation of, 229  
 Chrom-acetic solutions for plant cells, 217  
 Chrom blue GCB, 581  
 Chrom-osmo-acetic fluids, 218  
 Chrom violet, 587  
 Chromatin, 210  
 Chromatophores, demonstration of, 206  
 Chromic acid: for decalcification, 347  
   as fixative, 555  
   mixtures, 558  
 Chromomeres, relation of, to acidity of medium, 211  
 Chromosome constrictions, 208  
 Chromosomes: micrurgical study of, 99  
   plant, preservation of, 211  
 Chromotrope 2R, 581  
 Chromotrope 2R, 581  
 Chromotrope blue 2R, 581  
 Chrysoidin Y, 582  
 Chrysomonadina, 547  
 Churchill, H. R., 353  
 Cilia, methods for, 549  
 Ciliates: intestinal, 541  
   relief staining of, 549  
 Cladostephus, methods for, 233  
 Clark, E. R. and E. L., pipettes for micro-injection, 52  
 Clasmatoocytes, supravital staining of, 129  
 Clasmatodendrosis, 491  
 Cleaning: of glass in micrurgy, 77  
   of slides, 27  
 Clearing, 13, 26, 256  
   with aniline, 14  
   medium for, 13  
   time of, 14  
 Coccidia, 542  
 Cochineal, 573  
 Cold knife sectioning, 652  
 Cole, E. C., 479  
 Cole's rapid iron hematoxylin method, 630  
 Cole's stock hematoxylin solution, 631  
 Collagen, 404  
   fixation of, 404  
   silver impregnation, 406  
   staining of, 405  
 Collenchymatous walls, cutting of, 157  
 Collodion as a medium, 4  
 Collodion: method, 29  
   modifications of, 31  
   for plant tissues, 180  
   rapid method for nervous tissues, 446  
   sections: cutting of, dry, 31  
   for cytology, 261  
   handling, 30  
   staining of, 31  
 Colloidal chemistry, micropipettes in, 106  
 Colloidal lampblack for injection, 114  
 Colloidal suspension: of benzidine dyes  
   for injection, 115  
   of particulate matter for injection, 113  
   of silver and gold for injection, 115  
 Colophonium, 618  
 Color effects, 575  
 Color filters, 616  
 Color index, 302, 303  
 Combinations of agents as fixatives, 556  
 Comparison of control and incinerated sections, 657  
 Condenser for micrurgical technique, 72  
 Cone, William, 489  
 Congo blue 3B, 602  
 Congo corinth G or GW, 584  
 Congo red, 582  
 Conklin's modification of Delafield, 614  
 Conklin's modification of Wirtz' method, 141  
 Conn, H. J., 132, 573  
 Conn's stain for slime-forming bacteria, 146  
 Connective tissue,  
   attachments in muscle, 431  
   cells, 336, 337  
   supravital staining of, 128, 129, 130  
   intercellular substances of, 402  
   spreads, fixation and stains for, 337  
 Contractile vacuoles, 550  
   Protozoan, intra-vitam staining of, 527  
 Cooper method, 140  
 Copper salts for fixing Protozoa, 529  
 Corallin red, 582  
 Corallin yellow, 582  
 Coreine 2R, 582  
 Corinth brown G, 584



- Corn blue B, 602  
 Corn blue BN, 602  
 Corn syrup for plant tissues, 199  
 Correction for cell volume, 312  
*Corydalis cornutis*, 428  
 Cotton blue, 577, 582  
 Cotton corinth C, 584  
 Cotton red 4B, 579  
 Counterstaining, 26  
 Counting chambers, 288  
 Cover-glass preparations: handling of, 626  
     special method (Isaacs), 34  
 Cowdry, E. V., 246, 265  
     special methods, 265  
     for mitochondria, 267  
 Cox, W. H., 447  
 Cox-Golgi method, 448  
 Craigie, E. H., 471  
 Cresol red, 582  
 Cresol violet, 582  
 Cresolphthalein, 582  
 Cresyl blue 2N or BBS, 580  
 Cresyl fast violet, 582  
 Cresylecht violet, 582  
 Criteria of plant cytological methods, 204  
 Croceine scarlet, 579  
 Cryostat, 648, 650  
 Crystal violet, 137, 138, 583, 587, 593  
     and orange G, 190  
 Crystals: mounting of, 158  
     in plant cells, 221  
*Culex*, chromosomes in, 252  
 Culture plate, direct examination on, 133  
 Curcumine, 580  
 Cutin, staining of, 196  
 Cutler, puncture method of, 310  
 Cutting paraffin sections, 18  
     adjusting knife for, 18  
     mounting specimen for, 18  
 Cyanin and erythrosin, 193  
 Cycadales, methods for, 242  
 Cystoliths, 158, 222  
 Cytochemistry, enzymatic, 106  
 Cytological fixation, criteria for, 209  
 Cytological fixatives, method of application, 252  
 Cytological material: plant, choice of  
     method for, 204  
     preparation of, for fixation, 213  
 Cytological methods, general, 246, 248  
     for plant tissues, 204, 226  
     special, 265  
 Cytoplasmic inclusions: in muscle, 431  
     in plant cells, 220  
     in Protozoa, intra-vitam staining of, 528  
 Da Fano's cobalt nitrate silver method,  
     275  
     for Golgi net, 467  
 Dahlgren, Ulric, 420  
 Dahlia, 118, 588  
 Dahlia B, 593  
 Damar balsam, 618  
     for plant tissues, 200  
 Dare method, 296  
 Dark brown salt R, 582  
 Dark field: for incinerated preparations,  
     657  
     for micrurgy, 95  
 Davenport's modification: of Cajal, 459,  
     462  
     of Marchi, 476  
 Decalcification, 621  
     for Cajal's silver technic, 459  
     of teeth, 360  
 De Castro, F., 453, 455, 459  
 Decerating, 23  
 Deetjen - Weidenreich      agar - osmium  
     method, 332  
 Dehydration, 12, 26, 253  
     apparatus for, 254  
     of frozen tissue, 647  
     methods of, 12  
     temperature of, 647  
 Delafield's hematoxylin, 24, 613  
     for plant tissues, 186  
 Dental histological technique, 353  
 Dental periosteum, intravital methods  
     for, 389  
 Dental tissues, technique for, 353  
 Dentine: innervation of, intravital meth-  
     ods for, 389  
     method of Van Huysen, Hodge, Warren  
     and Bishop, 383  
     and pulp, methods for, 361  
 Dependorf's method for innervation of  
     pulp, 389  
 De Renyi, celluloid chamber of, 100  
 De Rivas' concentration method for Proto-  
     zoa, 540  
 Desiccation, 552  
 Desmids, fixation of, 228  
 Destin's fluid, 559  
 Dextrin for imbedding of teeth, 371  
 Diamin Bordeaux CGN, 584  
 Diamin red 4B, 579  
 Diamond fuchsin, 586  
 Diamond green, 591  
 Dianil blue 2R, 583  
 Dianil blue H3C, 602  
 Dianil red 4C, 579  
 Dianthin B, 584

- Dianthin C, 585  
 Diaphanol, 161  
 Diatoms: fossil, preparation of, 233  
     isolation of, 233  
     preparation of, 230  
 Diazin green, 589  
 Diethyl safranin, 119  
 Diffraction method: for measurement of  
     red blood cells, 314  
     for teeth, 381  
 Diffraction micrometers, 314  
 Diffusion of salts in fixation, 646  
 Dimethylamidoazobenzol, as a fat stain,  
     225  
 Dinoflagellates, 548  
 Dioxan, 38, 162  
     fixatives, 39  
     mounting medium, 39  
     preserving specimens in, 39  
 Direct garnet R, 584  
 Direct method for platelets, 295  
 Direct red, 582  
 Direct red 4B, 579  
 Direct steel blue BB, 583  
 Direct violet C, 584  
 Dissociated material, staining of, 608  
 Dissociating fluid, 439  
 Dobell's alcoholic hematein for Protozoa,  
     535  
 Dobell's picro-acetic, 534  
 Dominici's stain, 340  
 Dorner's method, 140  
 Double fixation, 250  
 Double green, 592  
 Double scarlet, 579  
 Downey, Hal, 324  
 Dry color films, preparation of, 118  
 Dry film method, 119  
 Drying: sections, 22, 27  
     spread sections, 22  
     test for completion of, 22  
 Dubreuil's iron hematoxylin method, 271  
 Duckwall's flagella stain, 142  
 Dust contamination, 652  
  
 Eckerson, Sophia, 155  
 Efflorescence of ash residue, 656  
 Egg, grasshopper, special method for, 40,  
     261  
 Ehrlich's aniline-fuchsin method, 153  
 Ehrlich's indulin-aurantia-eosin, 342  
 Ehrlich's intravital methylene blue, 477  
 Ehrlich's triacid, 330  
 Einarson's method for Nissl bodies, 451  
 Elastic fibrils, 413  
 Elastic tissue, 433  
  
 Electric organ tissues, 420  
 Electric tissues: methods for, 434  
     nerve connections in, 435  
     nerve endings in, 436  
 Electoplax: cytoplasmic inclusions of, 435  
     stains for, 435  
 Eleioplasts, demonstration of, 206  
 Embryological material: living, 281  
     prepared, 282  
     securing, 279  
     sources, 279  
 Embryological methods, 279  
     microtechnique in, 97  
 Embryo-sac, fixation of, 210  
 Embryos: affixing to paper, 284  
     arranging sections of, 284  
     chick, 280  
         living, 281  
         cleared, 284  
     dissection of, 283  
     infiltration schedule for, 283  
     injected, 283  
     living, 281  
     microinjection of, 283  
     mouse, 280  
     planes of sectioning, 285  
     sections, 284  
         serial, 284  
         thickness of, 286  
 Emerald green, 591  
 Emerson micromanipulator, 67  
 Enamel: v. Beust's method for, 373  
     chemical structure and composition of,  
         372  
     intra-vital dyes for, 375  
     Marshall's method for, 377  
     sodium alizarin-sulphonate for, 375  
     staining in bulk, 375  
     structure and chemical composition of,  
         372  
     William's method for, 374  
 Enamel rods, isolated, 372  
 Endophytic material, preparation of, 227  
 Entomostraca, muscle of, 421  
 Envelopes, slide, 28  
 Eosin: B, BN, BW, DKV, J, S, W or WS, Y, 584  
     alc. sol., 584, 592  
     bluish, 584  
 Eosin-methylene blue, for Protozoa, 536  
 Eosin scarlet B or BB, 584  
 Eosinophiles, vital staining of, 125  
 Epidermis, plant, maceration of, 173  
 Epiphytic material, preparation of, 227  
 Epithelial cells, vital staining of, 112  
 Equisetum, methods for, 241  
 Erie garnet B, 584

- Erythrosin B and BB, 584  
 Erythrosin BB, 598  
 Erythrosin bluish, 584  
 Erythrosin and cyanin, 193  
 Erythrosin R or G, 585  
 Erythrosin yellowish, 585  
 Ethereal oils in plant cells, 223  
 Ethyl eosin, 585  
 Ethyl green, 580  
 Ethyl purple 6B, 585  
 Ethyl violet, 585  
 Euglenoids, 548  
 Eutectic point, 647  
 Excelsior brown, 579  
 Extraction of lipoids, 647
- Fahraeus' method, 309  
 Farmer's fluid for plant cells, 216, 218  
 Farrant's medium, 617  
 Fasoli and Arlotta method, 393  
 Fast acid blue R, 585  
 Fast acid green N, 585, 590  
 Fast blue B, 585  
 Fast blue 3R, 585, 590  
 Fast fuchsin G, 582, 585  
 Fast green FCF, 585  
 Fast printing green, 586, 595  
 Fast red, 577, 586  
 Fast red B or P, 579, 586  
 Fast violet, 586, 587  
 Fast yellow, 586  
 Fat blue B, 602  
 Fat blue 4R, 602  
 Fat in plant cells, 222  
   staining of, 223  
 Fats: saponification of, 225  
   solubility of, 222  
 Fawcoksky's application of Cajal's silver  
   technic, 457  
 Feeding, effect of, on tissues, 247  
 Ferric chloride hematoxylin, Mallory's, 613  
 Fett Ponceau C, 600, 601  
 Feulgen reaction, 629  
   plant cells, 193  
 Fibers, study of, with micromanipulator,  
   106  
 Fibroblasts, supravital staining of, 130  
 Fibroglia fibrils: fixation of, 402  
   staining of, 403  
 Filamentous algae, 159  
 Filamentous organisms, 163  
 Filtering solutions, 616  
 Fischer, modification of Preiswerk's  
   method, 393  
 Fish embryos, Knowler's method of inject-  
   ing, 57
- Fitz's microinjector-aspirator, 92  
 Fitz's micromanipulator, 66  
 Fixation: chemical agents for, 552  
   collagen, 404  
   criteria of, 8  
   cytological, 218  
   double, 250  
   duration of, 250, 566  
   effect of temperature on, 564  
   fibroglia, 402  
   by immersion, 565  
   influence of physical conditions upon,  
     564  
   by injection, 566  
   for microincineration, 646  
   period of, 253  
   physical conditions during, 253  
   process of, 8  
   relation of staining to, 571  
   size and density of specimen, 564  
   temperature, 10  
   time of, 10  
   by vapors, 331, 562  
   vitaly stained tissues, 116  
 Fixation: and fixatives, 552  
   and staining, 266  
 Fixative: for plant cells, choice of, 215  
   injection of, 252, 568  
 Fixatives: chemical, 552  
   choice of, 8, 248  
   concentration of, 564  
   cytosomic, 563  
   differential effects of, 562  
   methods of application, 570  
   mordanting action of, 570  
   nuclear, 563  
   penetration, rate and degree of, 563  
   physical, 552  
   relative volume of, 565  
   selective action of, 562  
   temperature of, 564  
   washing out of, 569  
 Fixing, 8  
 Flagella: demonstration of, 205  
   stains, 141  
 Flagellates, intestinal, 541  
 Flemming's fluid, 249, 428, 433, 434, 559,  
   563  
   for nervous tissues, 469  
   for plant cells, 216  
 Flemming's tri-color stain, 192, 610  
 Flexner's method for leprosy bacilla, 154  
 Florey's technique, 95, 96  
 Fluorescein, 586  
 Fluorescence, 660  
 Fluorescent blue, 599

- Food vacuoles in ciliates, 527  
Foot, Nathan Chandler, 110  
Foot and Strobell, 258, 264  
Foot's modification of Bielschowsky's method, 410, 466  
Foot's modification of Cajal technic, 463  
Foot's modification of Hortega, 411  
Foraminifera, 546  
Formalin, 620  
Formic acid, 346  
    as fixative, 553  
Formol: as a dissociator, 439  
    as fixative, 554  
    for nervous tissue, 441, 444  
Formol-nitric solution, 559  
Formol-sublimate, 557  
Fossil plant material, grinding of, 174  
Free ends on muscle tissue, 421  
Free-hand microinjection, 51  
Free-hand sectioning of plant tissues, 176  
Freeman, G. L. *See* Papez, 476  
Freezing and drying tissues, 651  
Freezing method of sectioning, 32  
    for plant tissues, 178  
Freifeld's stain, 331  
Fritsch's method for innervation of dentine, 391  
Fry, H. J., 623  
Fuchsin: acid, 586  
    basic, 139, 142, 143, 146, 586  
    Mallory's acid, 404  
Fuchsin NB, 595  
Fuchsin S, SN, SS, ST, or SUI, 586  
Fucosan, 221  
Fucus, methods for, 233, 236  
Fungi, preparations of, 236  
Fused quartz rods: characteristics of, 632  
    method of illuminating, 632  
  
Gage's formol dissociator, 439  
Gage's method for glycogen, 468  
Gallamin blue, 587  
    for Nissl bodies, 452  
Gallocyanin, 587  
    for Nissl bodies, 451  
Gambine, 595  
Ganglia, silver method for, 454  
Garven's modification of gold-chloride technic, 477  
Gases, use for incineration, 654  
Gasteria, 167, 168  
Gatenby, J. B., 277  
Gelatin drop cultures, 133, 134  
Gelatin method for plant tissues, 178  
Gentian blue, 600  
Gentian violet, 137, 140, 583, 587, 593  
    Newton's, 171  
    and orange G, 190  
    and safranin, 189  
Gibson, William C., 481  
Giemsa stain, 328, 588  
    for bacteria, 150  
    for Protozoa, 536, 542, 543  
Gies' method, 376  
Gill plate, 36  
Gilson's fluid, 557  
    for plant cells, 216, 219  
Gilson's rapid collodion process, 31  
Gingiva, innervation of, 395  
Ginkgoales, methods for, 242  
Glass for micrurgical technique, cleaning  
    of, 77  
Glass slip: preparing for sections, 21  
    size of, 285  
Globus modification of Cajal, 500  
Glycerin jelly, 617  
Glycogen, 221  
    in nerve cells, 468  
Gold chloride method: for hemoflagellates, 545  
    for nerve endings, 477  
    for Trypanosomes, 545  
Gold chloride toning, 454, 464, 466  
Gold orange, 592, 596  
Gold sublimate method: principles of, 500  
    routine use of, 502  
Golgi apparatus, 274  
    experimental error in revealing, 537  
    in nerve cells, 467  
    in Protozoa, methods for, 537  
Golgi-Cox, 448  
Golgi method, 446  
    for dental periosteum, 390  
    for neurons, 445  
    modifications of, with formol, 448  
    rapid, 446  
    silver, 445  
Goodpasture's stain, 332  
Gottlieb's madder feeding method, 375  
Gower's solution, 287, 323  
Graham's benzidine stain, 333  
Gram's stain, 138  
    Hucker's modification of, 138  
    Kopeloff and Beerman's modification of, 138  
Gram-Weigert method, 152  
Grasshopper eggs, methods for, 40, 261  
Gray R, B, BB, 596  
Gray's flagella stain, 143  
Green algae, preservation of, 228  
Green PL, 595

- Gregarinidae, 541  
 Grieve's stain, 351  
 Griffithsia, methods for, 234, 235  
 Grinding, 4  
   method of, 37, 353  
   for fossil plant material, 174  
 Grüber's tannin mixture, 434  
 Gurdjian's modification of Cajal technic, 460  
 Gymnarchus, 434  
 Gymnosperms, methods for, 242  
  
 Haden-Hausser method, 297  
 Haden's method, 305  
 Hammerschlag's method, 306  
 Hance, R. T., 246  
   method for mammalian and avian tissues, 250  
 Hand microtome sectioning of plant tissues, 176  
 Hanging drop: for bacterial cultures, 133  
   cleanliness of, 90  
   of Protozoa, 525  
 Hardening, 10  
 Hart's modification of Weigert's elastic tissue stain, 414  
 Harvey, Ethel B., 615, 666  
 Hatai's method for centrosomes, 469  
 Hayem's solution, 287, 323  
 Hayne's phloxine-azure, 341  
 Heat, 552  
 Heidenhain's hematoxylin and eosin, 188  
 Heidenhain's hematoxylin for plant tissues, 167, 187, 190  
 Heidenhain's iron hematoxylin, 613  
 Heidenhain's trichloroacetic with sublimate, 557  
 Heldt, T. J., 449  
 Helianthin, 592  
 Heliotrope, 577  
 Heliozoa, 546  
 Helly's fluid, 121  
   for plant cells, 219  
   Zenker-formol, 337, 558  
 Helvetia blue, 592  
 Hemacytometer slides, 290  
   cleaning, 292  
 Hemagglutination and hemolysis, 314  
   measurement of, 317  
 Hemalum, Mayer's, 24  
   acid, 613  
 Hematein, 575  
 Hematein ammonium for fibroglia fibrils, 403  
 Hematocrit method, 303  
 Hematoxylin, 24, 574, 575  
   alcoholic, 535  
   Cole's, 631  
   Conklin's modification, 614  
   Delafield's, 24, 613  
   Heidenhain's, 610, 613  
   for plant tissues, 167, 187, 190  
   Kultschitsky, 472  
   lithium, 470  
   Weigert, 471  
   Mallory's phosphomolybdic acid, 406  
   Mallory's phosphotungstic acid, 403  
   for nerve fibers, 473, 474  
   rapid method of Cole, 630  
 Hematoxylin-balsam smear method for plant tissues, 166  
 Hematoxylin and eosin, 188  
 Hematoxylin and hematein, 613  
 Hematoxylin and safranin, 187  
 Hematoxylin smear method, 209  
 Hemipteran and Orthopteran germ cells, smear method for, 33  
 Hemoflagellates, 542  
 Hemosporidia, 545  
 Hemoglobin: amount of, 320  
   estimation of, 297, 299, 301, 320  
   correction for barometric pressure, 301  
   corrections for dissolved gas, 302  
   manometric method of, 301  
 Hemoglobin percentage, method of estimating, 295  
 Hemosporidia, 545  
 Herbarium specimens, preparation of, for microscopical study, 158  
 Hermann's platino-aceto-osmic acid mixture, 560  
 Hermetic chamber, 76  
 Hess and Zürcher, method of, 394  
 Heuser, C., 284, 615  
 Heuser injection method, 60  
 Heuser orientation methods, 622  
 Hexamethyl violet, 583  
 Higgins' ink for injection, 114  
 Hirschler's osmium method, 276  
 Hiss' method, 145  
 Histochemical reactions, 644  
   absorption in, 644  
   crystal formation in, 644  
   salt diffusion in, 644  
 Histochemistry, 643  
 Histological methods, plant, 156  
 Hörstadius, Sven, 43  
 Hofmann's green, 589  
 Hoffman violet, 588

- Hollande's fluid, 534  
     for flagella, 547  
     for Protozoa, 547
- Holmgren, trophospongium of, in nerve cells, 469
- Homarus, 431
- Hortega: 1927 method, 514  
     ammoniacal silver carbonate solution, 521  
     first variant, modification of, 509  
     formalin-iron-alum solution, 521  
     fourth variant, mordant for, 521  
     method  
         for astrocytes, 505  
         for gliosomes and mitochondria, 516  
         for microglia, 512  
         for neuroglia pigment, modification of, 516  
         for oligodendroglia, 510  
         for perivascular glia, 515  
         silver carbonate method, 520  
         Foot's modification, 411  
         uranium-formalin solution, 521
- Hoyer, H.: injection methods, 51  
     thionin method for mucin, 416
- Hrdlicka, A., 444
- Huber and Heuser, methods for thin sections, 623
- Huber's method: for cutting paraffin sections, 623  
     for Nissl bodies, 450
- Huggins, McCarroll and Dahlberg method for autogenous grafting, 397
- Huntoon's method, 146
- Hydrogen peroxide, bleaching method, 623
- Hydrophilus, 431
- Hydroquinone for reducing, 454
- Hyposulphite in toning, 454
- Ice formation in protoplasm, 649
- Illuminant: for micrurgy, 73  
     in relation to stain, 616
- Imbedding, 3  
     collodion, 16  
     frozen tissue, 32  
     paraffin, 16, 179, 251  
     watch-glass method, 16
- Imbedding and hardening in collodion, 29
- Imbedding and staining plant tissues, 220
- Imperial yellow, 578
- Impregnations, 461, 465, 609
- Incineration: oven, 653, 654, 655  
     preparation for, 651  
     process, 653, 655
- India ink method for dentine, 379
- Indin blue 2RD, 595
- Indicators, 102
- Indigo, 589
- Indigo blue, 589
- Indigo carmine, 589
- Indigotine 1A, 589
- Indophenol, 224
- Indophenol blue, 589
- Indulin: spirit sol., 589  
     water sol., 589
- Indulin black, 596
- Indulin-aurantia-eosin, 342
- Indusia, methods for, 241
- Infiltrated material, 4
- Infiltration, 14  
     collodion, 29  
     gradual, 257  
     paraffin, 14  
         with high temperatures, 258  
     schedule for embryos, 283
- Infusoria, 548
- Injection: apparatus for, 254  
     control of, 51  
     of fixatives, 252  
     gross, 51  
     mechanical, 53  
     pressure for, 51  
     steps in, 567  
     through the heart, 569
- In toto staining, 12, 607
- Intercellular substances in connective tissues, 402
- Interglobular spaces, demonstrating, 386
- Internodes of nerves, 441
- Interstitial cells: of central nervous system, 489  
     routine study of, 495
- Interstitial injections, 121
- Intestinal amebae, 540
- Intestinal ciliates, 541
- Intestinal flagellates, 541
- Intestinal Protozoa, 526, 538
- Intravital dyes for enamel and dentine, 375
- Intra-vitam dyes, 607
- Intra-vitam methylene blue method, 477
- Intra-vitam staining: plant cells, 212  
     Protozoa, 527
- Inulin, 158, 221
- Invertebrates, silver methods for, 454, 458, 459
- Iodine, for killing Protozoa, 529
- Iodine green, 589  
     and acid fuchsin, 189
- Iodine reaction for amyloid, 418

- Iodine violet, 588  
 Iodo-eosin B, 584  
 Iodo-eosin C, 585  
 Iris blue, 599  
 Iris violet, 577  
 Iron, 658  
 Iron alum, 619  
 Iron artifacts, 658  
 Iron hematoxylin: Heidenhain's, 25, 613  
     von Moellendorff's, 613  
     myofibrils, 426  
     for Protozoa, 534  
 Isaacs, Raphael, 287  
 Isaacs, Brock and Minot, method of, 308  
 Isamine blue, 589  
 Isolation of nerve cell bodies and fibers, 439  
 Iso-pentane in freezing, 649  
 Isorubin, 595
- Jansky method, 314, 316  
 Janus green, 117, 118  
 Janus green B, 266, 589  
 Jenner's stain, 328, 590  
 Johnson, silver method for degenerating nerves, 458  
 Johnston and Mack's flagella stain, 144  
 Johnston's method, 357  
 Johnston's neutral red stain, 450  
 Jones, Ruth McClung, 279  
 Juel's fluid, 560
- Kappers, Ariens C. U., 472  
 Kardos stain, 329, 340  
 Kardos-Pappenheim stain, 329  
 Kaufmann's method, 170  
 Keefe's fluid, 160  
 Khotinsky's cement, 90  
 Killing, 6  
 King, Helen Dean, 444  
 King's carbol thionin stain, 450  
 Kingsley's stain for sections, 335  
 Kirkman's neutral red stain for nerve cells, 450  
 Kleinenberg's fluid, 562  
 Knife, inclination of, 18  
 Knisely, M. H., 615, 632  
 Knower, H. McE., 51, 615, 630  
 Knower's method: for injecting fish embryos, 57  
     for injecting Amphibian larvae, 57  
     of microinjection, 55, 57  
 Koch-Weil method of making sections of teeth, 358  
 Kofoid and Swezy's alcoholic hematoxylin for Protozoa, 535
- Kolatchew's method, 276  
 Kopac, M. J., 62  
 Kopac's method for studying plasma-lemma, 95  
 Kopeloff and Beerman modification, 138  
 Kopsch's method, 276  
 Kopsch's modification of Golgi method, 418  
 Krause's membrane, 427  
 Krause's method, 350  
 Kristenson solution, 322  
 Kulp's flagella stain, 142, 144  
 Kultschitsky's hematoxylin, 472  
 Kuwada and Sakamura, 211
- Labeling slides, 28  
 Lacmoid, 590  
 LaCour fixing fluid, 219  
 Lactic acid, for decalcification, 347  
 Lactic-glycerin mounting medium, 245  
 Lacto-phenol mounting medium for plant tissues, 198  
 Lacunae and canaliculi, 349  
     staining linings of, 350  
 Lake Ponceau, 598  
 Lamellae, isolation of, 348  
 Landis' microinjection apparatus, 95  
 Langhans' method for amyloid, 418  
 Langworthy's intra-vitam methylene blue method, 479  
 Larsell's intra-vitam methylene blue method, 480  
 Latex: preservation of, 159  
     staining of, 194  
 Lauth's violet, 601  
 Lead, 660  
 Leather brown, 579  
 Leather yellow, 598  
 Leaves and stems, methods for, 241  
 Lefevre watch glass, 615  
 Leishman's stain, 326  
     for Protozoa, 544  
 LeMasurier, H. E., 443  
 Leucemic blood, supravital staining of, 127  
 Leucocytes, 324  
     fixation of, 337, 338  
     staining of, 339  
     vital, 111  
 Leucosin, 221  
 Lichens, methods for, 239  
 Liesegang's method for frozen sections of nervous tissue, 461  
 Light conduction in quartz rods, 636  
 Light filters, 211  
 Light green, 592

- Light green 2G, 3G, 4G, or 2GN, 590  
 Light green N, 591  
 Light green SF, yellowish, 590  
 Light source: for quantitative estimation of ash, 665  
     for quartz rod, 638  
 Lighting system for micrurgical technique, 73  
 Lignin, staining of, 195  
 Lillie's chrom-osmic-acetic mixture, 560  
 Linzenmeier method, 309  
 Lipoids, extraction of, 647  
 Liquid air, freezing in, 647  
 Lithium carbonate, for removing fixative, 11  
 Lithium carmine for injection, 115  
 Litmus, 590  
 Living cells, plant, examination of, 211  
 Living embryological material, 212  
 Living material, study of, 281  
 Locke's solution for Protozoa, 527  
 Loeffler's alkaline methylene blue, 137  
 Loeffler's flagella stain, 142  
 Loewitt method, 391  
 Ludford, R. J., 277  
 Lugol's solution, 529  
 Luminous organs, Lampyridae, 424  
 Lundsgaard and Möller method, 299  
 Lustgarten's solution, 472  
 Lymph nodes: fixation for, 337  
     stains for, 339  
 Lymphatics of tooth pulp: methods for injecting, 387  
     Dewey and Noyes' method for, 387  
     Schweitzer's method for, 387  
 Lymphocytes, vital staining of, 125  
 Lyons blue, 600  
  
 MacCallum's Goodpasture's method, 152  
 MacDaniel's method for intestinal Protozoa, 539  
 Macerated muscle tissues, 422  
 Macerating, 5, 37  
 MacNeal's tetrachrome stain, 325, 327, 601  
 Macro-method for determining blood cell volume, 303  
 Macroscopic staining of nervous tissue, 442  
 Madder feeding, Gottlieb's method, 375  
 Magdala red, 590  
 Magenta, 586  
 Magnesium citrate for decalcification, 348  
 Maier's sublimate alcohol, 533  
     for Protozoa, 533  
 Malachite green, 141  
 Malachite green G, 580  
  
 Malarial parasites, 545  
 Mallory, F. B., 132, 402  
 Mallory's acid fuchsin stain, 404  
 Mallory's aniline blue collagen stain, 405  
 Mallory's connective tissue stain for trophosphonium, 469  
 Mallory's eosin and methylene blue, 149  
 Mallory's ferric chloride hematoxylin, 613  
 Mallory's phosphomolybdic acid hematoxylin, 403, 406, 427, 613  
 Mallory's tricolor: for Protozoa, 535  
     Sharp's modification of, 535  
 Malone, E. F., 449  
 Malopterurus, 434  
 Mammalian cells, fixation of, 249  
 Mammalian embryos, injecting, 52  
 Manchester brown, 579  
 Manchester yellow, 591  
 Mandarin G, 596  
 Mann's methyl blue eosin, 536  
 Manson's methylene blue, 321  
 Marchi's method for degenerating myelin, 475  
 Marine blue, 577  
 Marine plant material, dehydration of, 164  
 Marking, 7  
 Marshall's method for tooth injection, 377  
 Martius yellow, 591  
 Mastigophora, 547  
 Material: cytological, selection of, 246  
     imbedded, 3  
     infiltrated, 4  
     physiological condition of, 247  
     to be stained  
         state of, 607  
         character of, 607  
     unsupported, 3  
 Mauvein, 118  
 Maximow, methods of, 337  
 May, method of, 310  
 May-Giemsa stain, 329, 340  
 May-Grünwald (Jenner's) stain, 328  
 May-Grünwald's fixation, 328  
 May's method, 310  
 Mayer: acid hemalum, 613  
     albumen, 620  
     amyloid stain, 419  
     carmalum, 612  
     chlorine method for bleaching, 623  
     hemalum, 613  
     muchematein method, 416  
     mucicarmin method, 417  
 McClendon, J. F., 565  
 McCord, Jolden and Johnston method, 321



- McClung, C. E., 3, 246, 279, 552, 573, 615  
 McClung's dioxan technique, 39  
 McDaniel's method for intestinal Protozoa, 539  
 McGill contraction nodes, 427  
 McNabb, Josephine W., 246  
 McNeil-Gullberg pipette, 90  
 Mean corpuscular hemoglobin: calculation of, 305  
     concentration, 305  
 Mean corpuscular volume, calculation of, 304  
 Medium, choice of, 4  
 Megacaryocytes and blood platelets, methods for, 333  
 Meiotic phases in plants, preparation of, 213  
 Meldola's blue, 595  
 Mercuric chloride, washing out, 11  
 Mercurochrome, 141, 591  
 Merkel's fluid, Smith's modification, 560  
 Meta-cresol purple, 591  
 Metanil yellow, 592  
 Methods: non-section, 5  
     for preparing material for microscopical examination, 3  
     of studying red blood cells, 287  
 Methyl blue, 592  
 Methyl eosin, 592  
 Methyl green, 592  
 Methyl-green-acetic, for Protozoa, 530  
 Methyl-green-pyronin, 341  
 Methyl orange, 592  
 Methyl red, 592  
 Methyl salicylate, 256  
 Methyl violet, 118, 587, 593  
 Methyl violet 10B, 583  
 Methylene azure, 593  
 Methylene blue, 118, 137, 139, 593  
     for study of dentine, 379  
 Methylene-blue-eosin, for Protozoa, 543  
 Methylene blue GG, 117  
 Methylene blue, intravital, 477  
     for staining isolated nerve cells, 439  
     for sections, 449  
 Methylene blue NN, 595  
 Methylene blue O, 602  
 Methylene blue, Unna's, 433  
 Methylene green, 594  
 Methylene violet, 594  
 Methylol, 162  
 Mettler's modification of Marchi's method, 475  
 Metz, C. W., 265  
 Meves' Victoria green method, 273  
 Microburner, 79  
 Microcautery, Péterfi, 86  
 Microchemistry, 106  
 Microcolorimeter, 102  
 Microelectrodes, 96  
     metallic, 96  
 Microdissections of embryos, 284  
 Microglia, 489, 494, 512  
     counterstaining of, 518  
 Micro-hydrogen electrodes, 96  
 Microincineration, 643  
     fixation of tissue for, 646  
 Microinjection: apparatus, blood capillary, 94  
     Chambers', 87  
     Brown's modification of, 54  
     Landis', 95  
     for renal corpuscles, 94  
     Taylor's, 90  
     Wearn and Richards', 94  
     White and Schmitt's, 94  
 apparatus and technique, 93  
     cellular, 86  
     directions for, 59  
     of embryos, 283  
     fixation of specimens, 61  
     fluids for, 59, 89  
     free-hand, 51, 53  
     partly mechanical methods for, 53  
     preparation of specimens for, 58  
     quantitative, 93  
     suggestions for, 57  
     under high powers, 54  
 Micromagnets, 96  
 Micromanipulative technique, 62  
 Micromanipulator, 69  
 Micro-method of determining blood cell volume, 303  
 Microneedles, 78  
     bending of, 82, 83  
     making of, 78, 79, 80, 81  
     mounting of, 84  
 Microorganisms, isolation of, 105  
 Micropipettes: glass for, 88  
     McNeil-Gullberg, 90  
     method of drawing, 52  
     mounting, 88  
 Micro-quinhydrone electrodes, 97  
 Microsaltbridges, 96  
 Microscope: inverted, 71  
     and microscope accessories, choice of, 70  
 Microscopic bacterial colonies, 133  
 Microscopical images, interpretation of, 9  
 Microsporidia, 551  
 Microtechnique in cytology and histology, 99

- Microtips: methods of obtaining, 86  
     opening of, 89  
     types of, 82
- Microtome, rotary, 178
- Micrurgical technique, 62  
     applications of, 97
- Micrurgical work, dark-field illumination, 95
- Mineral elements, demonstrations of, 643, 646
- Mitochondria, 118, 123, 125, 265, 578  
     fixation and staining, 266  
     in living cells, unstained, 265  
     methods for, 265  
     in nerve cells, 468  
     in Protozoa, methods for, 537
- Mitochondrial stains, Cowdry, 267
- Mitochondrial technique, experimental error in, 273
- Mixing fluids, agitation of, 254
- Mixtures, preparations of elements in, 625
- Moist chamber, 74  
     Reyniers, 77
- Mommsen's stain, 330
- Monocytes: pathological conditions of, 127  
     vital staining of, 126
- Monocytes and macrophages, supravital staining of, 129
- Moral, method of, 394
- Mordanting, 604
- Mordants, for hematoxylin, 575
- Morgan's myelin sheath stain, 473
- Mormyrus, 434
- Mounting, 27  
     incinerated preparations, 655
- Mounting media, 617  
     aqueous, 617  
         for plant tissues, 198  
     balsamic, 618  
     refractive index of, 197  
     Sandrac-Dioxan, 40
- Mounting paraffin sections, 19
- Mounting sections for incineration, 652
- Mouse uteri for blood vessels, 281
- Mozejko, injection methods of, 51
- Much's method, 139
- Muchaematein, Mayer's, 416, 614
- Mucicarmine, Mayer's, 417
- Mucilage, staining of, 196
- Mucin, 416
- Müller's fluid, 423, 442, 557
- Müller's method for Protozoa, 532
- Mulligan, J. H., 442
- Murray's method, 271
- Muscle, 420  
     fibers, 422  
     fixation of, 423  
     free ends of, 421  
     methods for, in contraction, 428  
     pressure ridges in, 421  
     section cracks in, 421  
     sectioning of, 426  
     smooth, 430  
     special characters of, methods for demonstrating, 421  
     staining of, 424, 426  
     striated, 428  
         frozen sections of, 423  
     teased, 422
- Muscle and electric organ tissues, methods for, 420
- Muscle cell, myogram of, 104
- Muscle material, sectioned, 423
- Mycorrhizas, endotrophic, preparation of, 239
- Myelin: degenerating, methods for, 475  
     formation in plant cells, 226  
     iron hematoxylin stain for, 473  
     normal, methods for, 469
- Myelin sheath: degenerating, 475  
     normal, 440, 469
- Myelinated nerve fibers, osmic acid methods for, 440
- Myofibrils: fixation of, 425  
     sectioning of, 426  
     smooth muscle, 427  
     stains for, 426  
         iron hematoxylin, 426
- Myxomycetes, methods for, 239
- Myxophyceae, preparation of, 228
- Myxosporidia, 550
- Nakamura, tooth germ transplantation, 399
- Naphthaline pink, 590
- Naphthaline red, 590
- Naphthamine blue 2R, 583
- Naphthamine blue 3BX, 602
- Naphthol blue, 595
- Naphthol green B, 595
- Naphthol green Y, 595
- Naphthol red, 577
- Naphthol yellow, 591
- Naphthylamine pink, 590
- Narcein, 595
- Navashin's fluid for plant cells, 216, 219
- Naysmyth's membrane, method for, 371
- Nebel, pollen tubes, 245
- Needham and Needham, 88

- Needle tips: Chabry, 86  
various, 86
- Needles, position of, in moist chamber, 85
- Nemec's fluid for plant cells, 219
- Nerve cells, isolation of, 439
- Nerve endings, 476  
by local application, 478  
staining by injection, 479
- Nerve fibers, isolation of, 439
- Nerves, Ranson and Billingsley's method for, 457
- Nervous tissue, removal of, 437
- Neubauer ruling, 288
- Neuroblasts, 454
- Neurofibrils, 452  
Cajal's method for, 452
- Neuroglia, 489
- Neurokeratin, 440
- Neurological technique, 437
- Neuromotor apparatus, 550
- Neuron shape and relationship, 445
- Neutral red, 117, 595  
reaction to intracellular materials, 118
- Neutral red iodide, 119
- Neutral red and Janus green mixture, 120, 122
- Neutral red stain for Nissl bodies, 450
- Neutral violet, 595
- New blue B, 595
- New fuchsin, 595
- New methylene blue N, 595
- New pink, 598
- New Ponceau 4R, 598
- New Victoria blue B, 602
- New Victoria green, 591
- Newcomer method, 296
- Newton's gentian violet, 171
- Niagara blue 3B, 602
- Nicholson's experiments in chromatolysis, 452
- Night blue, 600
- Nigrosin, 141
- Nigrosin w, wl, 596
- Nigrosin, water sol., 596
- Nile blue A, 596
- Nile blue sulphate, 596
- Nissl bodies, 449
- Nissl substance: degenerative, 452  
regenerative, 452
- Nitrazine yellow, 596
- Nitric acid: for decalcification, 346  
as fixative, 554  
mixtures, 559
- Noland's stain: for flagella and cilia, 530  
for Protozoa, 530
- Non-section methods, 5  
characteristics of, 6  
choice of, 6  
for plant material, 165
- Nopalin G, 584
- Nowlin's sublimate alcohol for Protozoa, 533
- Nuclear stain, 609
- Nuclei: of plants, fixation of, 209  
of Protozoa, methods of staining, 530
- Nucleolus, plant, fixation of, 209
- Nucleus and chromosomes, plant cell, 207
- Ocular micrometer, types of, 313
- Ohlmacher's modification of Carnoy's fluid, 444
- Oil of cloves method of mounting sections of teeth, 365
- Oil red, 600
- Oil red A, 224
- Oil red IV, 601
- Oil red O, 596
- Oil scarlet, 596
- Oil vermilion, 601
- Oligodendroglia, 491
- Omentum, 35
- Omentum and mesentery spreads, 336
- Orange A, P or R, 596
- Orange extra, 596
- Orange G, 597
- Orange G and gentian violet, 190
- Orange G and safranin, 189
- Orange G, safranin and gentian violet (Flemming's tri-color), 192
- Orange II, 596
- Orange III, 592
- Orange MNO or MN, 592
- Orcein, Unna's, 425, 597
- Orientation methods, 621
- Orseilin BB, 597
- Orth's fluid, 441
- Orthopteran cells, method for, 251
- Osgood-Haskins method, 297
- Osgood and Wilhelm, method of, 319
- Osmic acid: as a fat stain, 225  
for fixing Protozoa, 529
- Osmic acid mixtures, 559
- Osmic method for myelinated nerves, 440
- Osmium methods in cytology, 276
- Osteoblasts, processes of, 351
- Ottenberg and Rosenthal solutions, 322
- Ovaries, plant, fixation of, 214
- Oxygen, combining power of blood, 299
- P.F.A.-3 (fixing fluid), 561
- P.F.A.-15 (fixing fluid), 561

- P.F.A. mixtures, 561  
Pal modification of Weigert's method, 472  
Pal's decolorizer, 409  
Panchrome stain, 329, 340  
Papez's experiments in chromatolysis, 452  
Papez's modification of Marchi's technique, 476  
Pappenheim stain, 341  
Paraffin, 620  
    choice of, 15  
    filtering, 621  
    grade of, 15  
    infiltrating with, 14  
    infiltration, time of, 15  
    as a medium, 4  
    method, 6, 14  
        for plant tissues, 179  
        recent modifications of, 38  
    ribbons: arranging of, 22  
        expansion of, 22  
        manipulation of, 20  
        storage of, 21  
    rubber, 621  
    sections: cutting, 18  
        spreading of, 21  
    technique, for teeth, 360  
Paraffin oil, for plant tissues, 200  
Para-fuchsin, 597  
Para-magenta, 597  
Paramylum, 221  
Para-rosanilin, 597  
Parasolic acid, 597  
Paris blue, 600  
Paris violet, 593  
Parker, Frederic B., 132, 402  
Parowax, 258  
Particulate matter for injections, 113  
Pectin, staining of, 196  
Peeling method for fossil plant material, 175  
Pelecypod muscle, 430  
Penfield, W. C., 467, 489  
Penfield's method for oligodendroglia,  
    first modification, 510  
    second modification, 511  
Penfield's oligodendroglia and microglia  
    method, 511  
Perdrau's modification of Bielschowsky's  
    method, 409  
Perenyi's fluid, 559  
Perfusion with stain, 121  
Peritoneal fluid, methods for, 332  
Peroxidase reaction, 332  
Péterfi micromanipulator, 65  
Petrifaction method for tooth sections, 358  
Phaeophyceae, 233  
Phagocytes, vital staining of, 111  
Phagocytic cells and supravital dyes, 117  
Phenolphthalein, 597  
Phenol red, 597  
Phenylene blue, 595  
Phenylene brown, 579  
Phloxine, 590, 598  
Phloxine azure stain, 341  
Phosphine, 598  
Phosphomolybdic acid, Mallory's, 613  
Phosphoric acid for decalcification, 347  
Phosphorus, loss of, 658  
Phosphotungstic acid hematoxylin, Mallory's, 613  
Photoelectric cell in study of minerals, 663  
Photoelectrometer for hemoglobin estimation, 301  
Photographic method for mineral residues, 662  
Photometer ocular, 102  
Physical agents for fixation, 552  
Physical conditions in fixation, 564  
Pianese IIb for fungi, 238  
Picric acid: for decalcification, 347  
    for destaining hematoxylin, 548  
    as fixative, 555  
Picro-acetic mixture, 562  
    for Protozoa, 533  
Picro-carmin, 611  
Picro-formol-acetic, 561  
Picro-formol-acetic mixtures, 9, 248  
Picro-mercuric fixative for Protozoa, 534  
Picro-nigrosin, 425  
Picro-sulphuric acid mixtures, 562  
Pigment in nerve cells, 469  
Pijper's method, 314  
Pinacyanol, 598  
Piney's pipette, 288  
Pipette shaker, automatic, 289  
Pipettes: cleaning, 292  
    preparation of, 59  
Pith embedding, 178  
Plant, J. H., 443  
Plant cytological methods: choice of, 204  
    types of, 205  
Plant epidermis, maceration of, 173  
Plant histological methods, 156  
Plant material: dehydration and clearing  
    of, 161, 164  
    preparation of, 156  
    preservation of, 156  
Plant membranes, microchemical reactions in, 195

- Plant sections: dehydration of, 161  
 staining of, 184
- Plant tissues: decalcification of, 160  
 desilification of, 161  
 free-hand cutting of, 176  
 hand-microtome for, 176  
 microtome section of, 177, 178  
 preservation medium for, 159
- Plants, teasing method for, 173
- Plasma cells, supravital staining of, 130
- Plasma stain, 609
- Plasmodesmen, 205
- Plass and Rourke method, 311
- Platelet counting calculations, 294
- Platelet diluting solutions, 322
- Plathner precipitin reaction, 400
- Platinic chloride mixtures, 560
- Plimmer and Paine's flagella stain, 144
- Podophyllum, 168
- Polarized light, 658
- Policard, A., 644, 646, 652, 656, 658
- Pollen studies, methods for, 243
- Pollen tubes, methods for, 244
- Polychromatophilia, 320
- Polychrome stains for Protozoa, 535
- Ponceau B, 579
- Ponceau 3B, 601
- Ponceau 2R, 598
- Ponceau R, RG, B, 4R, 2RE, NR, J, FR, GR, 598
- Ponder and Millar method, 313
- Post-mortem stains, 607
- Potassium, 659
- Pratt's method for platelets, 295
- Pratt's solution, 322
- Precipitin reactions with bone and teeth, 400
- Precision needle holder, 84
- Preiswerk, method of, 393
- Prepared embryological material, 212
- Preservation of nervous tissue, 441
- Preserving fluids, 621
- Pressure ridges and section cracks, 421
- Processes of young osteoblasts, 351
- Progressive staining, 609
- Proportion of elements in fixatives, 556
- Protein in plant cells, 221
- Protoplasm: effects of micrurgical technique upon, 63, 64  
 pH of, 102  
 physical state of, 102
- Protozoa, 522  
 albumen smears, 530  
 anesthetization of, 526  
 associated forms, 526
- Protozoa—(Continued)  
 blood-inhabiting, 542  
 fixation of, 542  
 staining of, 543, 544  
 thick films of, 544  
 wet films of, 544  
 collodion and paraffin embedding of, 538  
 contractile vacuoles, 527, 550  
 ectozoic, 526  
 endozoic, 526, 538  
 fixation of, 531  
 fixing and staining on slip, 531  
 food vacuoles, 527  
 free-living, 522  
 handling in bulk, 531  
 intestinal, 538  
 concentration methods for, 540  
 examination of, 526  
 fixation of, 539  
 smears of, 539  
 staining of, 539  
 washing cysts, 539  
 intra-vitam staining, 527  
 killing and staining methods for, 528  
 living, collection of, 522  
 concentration of, 522  
 examination of, 522  
 mounting individual specimens of, 532  
 permanent mounts, 530  
 sectioning en masse, 537  
 sectioning individuals, 538  
 sectioning methods, 537  
 Sedgwick-Rafter method for, 523  
 slowing movements of, 526  
 staining methods, 534  
 permanent mounts, 534  
 temporary killing and staining of, 528  
 washing and dehydration, 532
- Protozoan collections, examination of, 525
- Protozoan cultures, examination of, 525
- Protozoological methods, 522
- Protozoology, microtechnique in, 97
- Pteridophytes, 240
- Pulp and dentine, innervation of: Dependorf's method, 389  
 Huber's method, 389
- Pulp canal, injection of, 374
- Pulp chamber, form of, 393
- Pulp chamber and canals: Adloff's method for, 393  
 Barrett's method for, 395  
 Fasoli and Arlotta's method for, 393  
 Fisher's method for, 393  
 Hess and Zürcher's method for, 394

- Pulp chamber—(Continued)  
 Moral's method, 394  
 Preiswerk's method for, 393  
 Pure cultures, study of, 133  
 Purpurin, 598  
 Pyoktanin blue, 593  
 Pyoktanin yellow, 578  
 Pyoktaninum aureum, 578  
 Pyramidal cells, 444  
 Pyrenoids, demonstration of, 206  
 Pyridine, as a fixative, 456  
 Pyrogallol as a reducer, 454  
 Pyronin B and Y, 118, 599  
 Pyrosin B, 584  
 Pyrosin J, 585
- Quantitative estimation of ash, 661  
 in normal pathologic tissues, 661  
 Quartz rod, parts of, 636  
 Quigley, B., 473
- Radiolaria, 546  
 Rainbow scarlet, 598  
 Ranson's modification of Cajal's method, 457  
 Ranson's pyridine silver method, 457  
 Ranvier and Cohnheim, innervation of  
 gingiva, 395  
 Ranvier's ammonia carmine, 611  
 Ranvier's dissociating fluid, 439  
 Ranvier's gold chloride for nerve endings,  
 477  
 Ranvier's nodes, 440  
 Rat brains, fixation of, 249  
 Reagents, 9  
 preparation of, 620  
 Reconstruction method, 286  
 Reconstruction process, 286  
 Red blood cells, 287  
 agglutination, 317  
 counting, 287  
 hemagglutination and hemolysis, 314  
 hypochromic, hyperchromic and or-  
 thochromic, 320  
 measuring, 311  
 diffraction method of, 314  
 photographic method of, 313  
 projection method of, 313  
 nuclear particles in, 321  
 refractive granules in, 321  
 resistance,  
 to heat, 308  
 to hypotonic salt solutions, 307  
 sedimentation rate, 309, 310, 311  
 sickling of, 318
- Red blood cells—(Continued)  
 staining,  
 of reticulum, 319  
 supravital, 122  
 stippling of, 320, 321  
 structures of, 319  
 suspension stability of, 309  
 washing, 305  
 Red blood counting pipette, 287  
 Red violet, 588  
 Rees and Ecker solution, 322  
 Rees' method for blood protozoa, 544  
 Refractive granules, 321  
 Refractory tissues, sectioning in paraffin,  
 40  
 Regaud's hematoxylin, 535  
 Regaud's iron hematoxylin method, 270  
 for Protozoa, 535  
 Regressive staining, 609  
 Relief stains, Protozoa, 544  
 Removal of nervous tissue from body, 437  
 Renal corpuscles, microinjection appa-  
 ratus for, 94  
 Renyi's celluloid chamber, 100  
 Resin, 222  
 in tissues, preservation of, 159  
 Resinous mounting media for plant tis-  
 sues, 200  
 Resins, staining of, 196  
 Resorcin blue, 599  
 Reticulum: of red blood cells, staining of,  
 319  
 of reticulocytes, 123  
 Retterer's stain, 424  
 Reyniers' microinjection apparatus, 92  
 Reznikoff, method of, 306  
 Rhodamine B, 599  
 Rhodamine O, 599  
 Rhodophyceae, methods for, 234  
 Ribbon, paraffin, 19  
 expansion of, 22  
 numbering, 21  
 segments, arranging, 22  
 splitting of, 19  
 static electricity in, 20  
 Ringer-formalin solution, 121  
 Ringer's solution for Protozoa, 526  
 Rio-Hortega, P., 469. *See also* Hortega.  
 de Rivas' concentration method, 540  
 Roger's modification of Bielschowsky's  
 method, 466  
 Romanowsky's stain, 326, 599  
 Root and stem tip, preparation of, 213  
 Rosanilin, 599  
 Rosazine, 578  
 Rose bengal, 146, 147, 599

- Rosinduline GXR, 578  
 Rosolic acid, 600  
 Ross, L. S., 469  
 Rotary microtome for plant tissues, 178  
 Rourke and Ernestine method, 311  
 Rubber paraffin, 258, 621  
 Rubin, 586  
 Russell's stain for Protozoa, 544  
 Rye as cytological material, 168  
  
 Sabin, Florence R., 117  
 Safranin, 138  
 Safranin and aniline blue, 189  
 Safranin and crystal violet for plant tissues, 189, 191  
 Safranin and fast green for plant tissues, 188  
 Safranin and hematoxylin, 187  
 Safranin and light green for plant tissues, 188  
 Safranin O, 600  
 Safranin and orange G, 189  
 Safrosin, 584  
 Sahli method, 295  
 Sandrac, as a mounting medium, 40  
 Saponification of plant fat, 225  
 Sarcodina, 545  
 Sarcolemma, 431  
     striated muscle, 432  
 Sarcosporidia, 551  
 Saturation index, calculation of, 305  
 Scarlet B or EG, 579  
 Scarlet C or B, 600  
 Scarlet J, JJ or V, 584  
 Scarlet R, 598  
 Scarlet red, 601  
 Schaeffer and Fulton, modification of Wirtz' method, 141  
 Scharlach red, 601  
 Schaudinn's fluid, 533  
     for plant cells, 219  
 Schmorl's method, 350  
 Schmuck, Louise, 265  
 Schmuck and Metz, chromosomes of entire eggs, 265  
 Schneider's aceto-carmin, 612  
 Schour's method for bone and teeth, 377  
 Schultz, Brauns O., 652, 654, 659  
 Schultz, O., modification of Cajal's technique, 462  
 Sciarra chromosomes, 265  
 Scott, Gordon H., 615, 643, 649, 650, 652, 657, 662  
 Section methods, 3  
     characteristics of, 5  
     Section and non-section methods of preparing microscopical slides, 3  
     Section thickness, 251  
     Sectioning: cytological, 258  
         plant tissues, 176, 177, 178  
         Protozoa, 537  
         woody tissues, 182  
     Sections: arrangement of, 20  
         bleaching of, 251  
         compression of, 19  
         cytological, with collodion, 261  
         dehydration of, 163  
         drying, 21  
         faults in, 19  
         hydrating, 24  
         irregularities in, 20  
         mounting, 21  
         of plant material, clearing of, 163  
         spreading of, 21  
         staining of, 608  
         of teeth,  
             examination with polarized light, 379  
             with reflected light, 385  
             with ultraviolet light, 381  
             with roentgen rays, 381  
         thickness of, 625  
             for embryos, 286  
 Sedgwick-Rafter method for Protozoa, 523  
 Sensitol red, 598  
 Sharp's modification of Heidenhain's method, 548  
 Shipley, P. G., 344  
 Shour and Smith, enamel and dentine, 378  
 Shrinkage during incineration, 653  
 Shrinkage and swelling effects, balancing, 556  
 Shunk's flagella stain, 142  
 Sieve tubes, 159  
     staining of, 194  
 Silica, 659  
     detection of, 196  
 Silver gray, 596  
 Silver impregnation methods: for ciliates, 549  
     for collagen, 406  
     for Golgi apparatus, 275  
 Silver nitrate methods: Bielschowsky's, 463  
     Cajal's, 452  
     Golgi's, 445  
 Silver oxide method for microglia, 515  
 Silver staining, general observations on, 503  
 Simple fluids as fixatives: advantages of, 554  
     disadvantages of, 554

- Sincke, G., 442  
 Size and density in specimens, 564  
 Sjovall's osmium method, 276  
 Slide envelopes, 28  
 Slider, Ethel N., 324  
 Slides: cleaning of, 27  
     labeling of, 28  
     manipulating, 24  
     storing of, 28  
 Sliding microtomes for cutting plant tissues, 177  
 Slips, cleaning, 21  
 Small objects: changing fluids on, 570  
     handling of, 615  
 Smear method, 33  
     advantages of, 34  
     cytological, 262  
     Foot and Strobell, 264  
     for plant tissues, 173  
 Smearing, 5, 165  
 Smears: blood, dry, 325  
     wet, 331  
     fixing, 33, 264  
     germ cell, 33  
     plant, 165  
     hematoxylin-balsam method, 166  
     Kaufmann's method for, 168  
     preparations, bacterial, 135  
     staining, 264  
     by wiping, 34  
 Smears on cover glasses, 33  
 Smears on slips, 34  
 Smith's modification of Newton's method, 171  
 Smith's myelin sheath stain, 473  
 Smooth muscle, 430, 433  
 Soap method for plant tissues, 179  
 Sodium, 659  
 Sodium-alizarin-sulphonate, 375  
 Solid green, 591  
 Soluble blue 3M or 2R, 577  
 Soluble yellow OL, 592  
 Solutions: of aniline dyes, strength of, 604  
     of solids as fixatives, 555  
     advantages of, 556  
     disadvantages of, 556  
 Solvents: of aniline dyes, 603  
     for stains, 605  
 Sosman, R. B., 632  
 Special dental structures, examination of, 371  
 Specimen, position of, in fixing, 565  
 Specimens: condition of, at fixation, 247  
     preliminary treatment of, 246  
     preservation of, 626  
     tinting, 627  
 Spencer bright line chamber, 288  
 Sperms in plants, staining of, 194  
 Sphecius, 431  
 Spinal cord: rapid method for staining, 630  
     reduced silver methods for, 453  
 Spinal cord and brain stem, rapid staining method, 630  
 Spireme in plant cells, 210  
 Spirit blue, 600  
 Spleen: fixation for, 337  
     stains for, 339, 340  
     Zenker-formol fixation of, 338  
 Spleen and lymph nodes, methyl-green-pyronin stain for, 341  
 Sporangia: fixation of, 215  
     methods for, 241  
     of bryophytes, fixation of, 215  
 Spore stains, 140  
 Sporelings and epiphytes, methods for, 235  
 Spores: methods for, 239  
     shrinkage of, 551  
 Sporozoa, 550  
     intestinal, 541  
 Spreading, test for completeness of, 259  
 Spreading sections, 21  
 Spreads, 336  
 Sprengel, Berkelbach von der, method of, 392  
 Staining, 24, 570  
     agents:  
         chemical composition of, 605  
         inorganic, 605  
         nature of, 573  
         organic, 573  
         origin or source of, 573  
         physical composition of, 605  
     capacity, revival of, 571  
     collagen, 405  
     collodion sections, 31  
     combinations, 605  
         physical nature of, 605  
     cytological, 260  
     dissociated material, 608  
     fibroglia, 403  
     by impregnation, 608  
     muscle, 426  
     plant tissues, 184, 185  
     preparation for, 24  
     progressive, 609  
     regressive, 609  
     sections, 608  
     stretched material, 608  
     time of, 25  
     in toto, 12, 607



- Stains: adjective, 608  
     alcoholic, for plants, 186  
     application of, 607  
     aqueous, for plants, 185  
     chemical nature of, 606  
     combination for plants, 187  
     composition of, 606  
     concentration of, 606  
     for fluid mounted material, 198  
     general, 609  
         bacterial, 136  
     inorganic, 605  
     intra-vitam, 607  
     mordanting for, 604  
     multiple, 606  
     nuclear, 609  
     physical composition of, 605  
     plasma, 609  
     post-mortem, 607  
     progressive, 609  
     regressive, 609  
     relation of: to fixative, 604  
         to character of material, 608  
     selective, 609  
     simple, 606  
     solvents for, 605  
     substantive, 608  
     temperature of, 606  
     in toto, 607  
 Stains and staining, 573  
 Starch, preservation of, 159  
 Starch grains, demonstration of, 220  
 Steel gray, 596  
 Stöhr, P., Jr., 622  
 Storing slides, 28  
 Streaming protoplasm, material for the study of, 100  
 Stretched material, 608  
 Stretching, 5  
     advantage of, 36  
     method, 35  
 Striated muscle, sarcolemma of, 432  
 Stromsten's fluid for plant cells, 219  
 Strong's modification of Golgi's method, 448  
 Subcutaneous tissue spreads, 336  
 Suberin, staining of, 196  
 Sublimate acetic, 557  
 Sublimate alcohol for Protozoa, 533  
 Sub-stage condenser, 73  
 Substantive stains, 608  
 Sudan c, 600  
 Sudan II, x or AX, 596  
 Sudan III, 600  
     for staining fat in plant cells, 223  
 Sudan IV, 601  
     for staining fat in plant cells, 224  
 Sudan R, 601  
 Sudan red, 590  
 Sugar in fixatives, 9  
 Sugita, N., 444  
 Sulphate formation, 660  
 Sulphur, 222  
 Sulphuric acid, 346  
 Sulphurous acid, 346, 623  
 Sultan 4B, 579  
 Supravital dyes, 117  
     fixation, 121  
     reacting to one substance, 118  
     reacting to several substances, 117  
 Supravital staining, 117, 119, 265  
     by immersion (Cowdry), 265  
     by injection, 266  
     methods of, 118  
         for basophiles, 125  
         for blood, 122  
         for cells from organs, 130  
         for cerebrospinal fluid, 128  
         for eosinophiles, 125  
         for leucemic blood, 127  
         for liver, 130  
         for lymphocytes, 125  
         for monocytes, 126  
         for red blood cells, 123  
         for white blood cells, 124  
 Supravital stains, application of, 122  
 Surface scums of cultures, 530  
 Sutro, C. J., 615, 671  
 Swank, R. L., 476. *See* Davenport  
 Swiss blue, 593  
 Synthesis in plant cells, 210  
 Takahashi's technic with osmic acid, 441  
 Tallqvist's method, 296  
 Tannin, 222  
 Taylor, William Randolph, 155  
 Taylor and Kaufmann, smear method, 263  
 Taylor's method, 168  
 Taylor's microinjection apparatus, 90, 91  
 Taylor's micromanipulator, 64  
 Taylor's modification of Flemming's fluid, 218  
 Teased muscle tissues, 422  
 Teasing, 6, 36  
 Teasing method for plant tissues, 173  
 Teeth: enamel cuticle of, 371  
     examination of,  
         by polarized light, 379  
         by reflected light, 385  
         by roentgen rays, 381

- Teeth—(Continued)  
frozen sections of, 371  
grinding of, 353  
household cement method, 356  
sections on wheel, 354  
preparation of, for grinding, 356  
sections of, 385  
study by ultraviolet light, 381  
whole, examination of, 353  
Tellyesniczky's fluid, 558  
Temperature and fixation, 10  
Temperature control, 650  
in quartz rod illumination, 639  
Temperatures for cytological infiltration, 257  
Tetrachrome stain, MacNeal, 325, 327, 601  
Thickness of sections, 625  
Thin sections, Huber and Heuser method, 623  
Thionin, 601  
Thionin, alcoholic, for mast cells, 342  
Thionin stain for Nissl bodies, 450  
Thoma pipette, 288  
Thymol blue, 602  
Tinting specimens, 627  
Tissue culture, micrurgical technique in, 101  
Toisson's solution, 287, 323  
Toluene red, 322  
Toluidine blue, 342, 343  
Toluidine o, 602  
Toning: after Bielschowsky's method, 464  
Cajal's method, 454  
Roger's method, 466  
Tony red, 600  
Torpedo, 434  
Tooth germ, development of, in vitro, 399  
Tooth germ element, transplantation of, 397  
Toxic neutrophiles, 330  
Tradescantia, 168, 212  
Trenner's pipette, 288  
Trephine method for bone marrow, 338  
Triacid stain, 330  
Trichites, 549  
Trichloroacetic acid, for decalcification, 347  
Trichocysts, 549  
Trioxymatein, 575  
Trojoda's method for innervation of dentine, 391  
Tropaeolin D, 592  
Tropaeolin G, 592  
Tropaeolin ooo no. 2, 596  
Trophospongium in nerve cells, 469  
Trypan blue, 602  
Trypan red, 602  
Tuan's destaining method, with picric acid, 548  
Tuan's modification of hematoxylin stain, 190  
Tuan's safranin and picric acid technique, 171  
Ultraviolet light, 657, 658  
Ultropak illuminator, 74  
Ultropak micromanipulator, 74  
Unna's methylene blue, 433  
Unna's orcein, 425  
for elastic fibers, 415  
Unsupported material, 3  
Uranin, 586  
Uranium, 660  
Uranium-formol method of Cajal, 467  
Urea in fixatives, 9  
Urethane, Castro's formula, 459  
Vacuoles of digestion, 117  
Vacuum system, 651  
Van Allen method, 303  
Van Gieson's picric acid and acid fuchsin, 405, 423  
Van Gieson's stain, 405  
Van Huysen, Hodge, Warren and Bishop, method for study of dentine, 383  
Van Slyke and Neill method, 301  
Van Slyke and Stadie method, 299  
Vapors as fixatives, 562  
Vascular endothelium, vital staining of, 112  
Vascular injection of dyes for supravital reactions, 120  
Vaseline for sealing, 123  
Velthemia, 168  
Venetian turpentine infiltration method, 203  
Verhoeff's elastic tissue method, 414  
Verhoeff's stain, 444, 613  
Verocay's stain for collagen, 406  
Veronal as a fixative, 455  
Vesuvium, 579  
Victoria blue B, 602  
Victoria blue R, 602  
Victoria rubin, 577  
Violamine 3B, 585  
Violet C, G or 7B, 583  
Violet R, RR or 4RN, 588  
Vital dyes, 117  
injection of, 120  
specificity of, 111  
Vital neutral red, 117  
Vital red, 603

- Vital staining: applicability of, 112  
 injection routes for, 110  
 technique of, 113  
 types of material used for, 111
- Vital stains, 110  
 combinations of, 116
- Volatilization of inorganic salts at heats of incineration, 657
- Volume of blood cells, method of determining, 303
- Volume index: blood, 304  
 method of obtaining, 304
- Volutin, 221
- Volvocidae, 548
- Von Möllendorf's iron hematoxylin, 613
- Walker's application of Cajal technic, 456
- Washing, 11, 253  
 adjuvants for, 11  
 duration and extent of action, 570  
 facilitation of, 11  
 after fixation, 569  
 medium for, 11, 569  
 after staining, 25  
 time of, 11
- Water blue, 577
- Water soluble eosin, 584
- Weigert's elastic tissue stain: Hart's modification, 414  
 Meyer's modification, 471
- Weigert's hematoxylin, 471  
 differentiating fluid, 470  
 modifications of, 471  
 myelin sheath method, 469  
 neuroglia mordant, 470
- Weigert's Mann-Kopsch method, 277
- Weigert's method for elastic fibrils, 413
- Weil's myelin sheath stain, 474
- Wenrich, D. H., 522
- Wenrich and Geiman's modified Schaudinn's fluid for Protozoa, 533
- Wet knife for paraffin sections, 623
- Wetzel's picro-formol-acetic, 533, 534
- White blood cells,  
 counting, 292, 293  
 supravital staining, 124
- White fibrous tissue, teasing, 36
- White's method for interglobular spaces, 387
- Whole mounts: of embryos, 282  
 time schedule for, 285  
 of fixed specimens, 282
- Widmark and Ørskov burette, 93
- Wilder's silver stain for reticulum fibers, 412
- Williams' silver nitrate method, 374
- Wilson's blood stain, 326
- Windle's experiments in chromatolysis, 452
- de Winiwarter, H., 250
- Wintrobe method of determining blood cell volume, 303
- Wirtz' method, 141
- Wolbach's Giemsa stain, 151
- Wolf quartz rod, 632
- Woods, maceration of, 174
- Woody material, preservation of, 159
- Woody tissues, sectioning, 40
- Wool orange G, 597
- Wool red, 577
- Worcester's fluid, 557
- Wratten and Wainwright filters, 616
- Wright and Kinnicutt solution, 323
- Wright's blood stain, 326, 603  
 for Protozoa, 543
- Wright's megacaryocyte stain, 334
- Wright's methylene blue azure, 117
- Writing fluids, staining Protozoa with, 529
- Xanthene, 598
- Xanthophyceae, preparation of, 230
- Xylidine Ponceau 3RS, 598
- Yellow elastic tissue, teasing method for, 36
- Yellow M, 592
- Yellow WR, 580
- Yocum's picro-mercuric, 534
- Yolk-laden eggs, sectioning, 40
- Zeckwer and Goodell method, 309
- Zenker-formol, 558
- Zenker's fluid, 423, 427, 434, 558
- Ziehl-Gabbet method, 139
- Ziehl-Neelsen method, 139
- Ziehl's carbol-fuchsin, 136